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How Understanding and Harnessing the Microaerobic Metabolism of Glycerol in *Escherichia coli* can Revitalize the Biodiesel Industry

By

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ABSTRACT

How Understanding and Harnessing the Microaerobic Metabolism of Glycerol in *Escherichia coli* can Revitalize the Biodiesel Industry

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Governments want to replace fossil fuels with biofuels like ethanol and biodiesel due to concerns about pollution and price instability. However, the biodiesel industry must find a market for glycerol to be profitable. The large quantities and reduced state of its carbon have made glycerol an attractive choice for ethanol production. While *Escherichia coli* can produce ethanol anaerobically, cell growth requires expensive complex nutrients. To bypass this issue, we used microaerobic conditions and maintained 91% recovery of carbon into products. We identified the pathways involved in the microaerobic metabolism of glycerol using genetic and biochemical tools and further engineered the optimized anaerobic *E. coli* strains for microaerobic conditions. Greater cell growth and product synthesis were achieved by overexpressing glycerol dehydrogenase and dihydroxyacetone kinase. The final strain *ΔfrdAΔptaΔldhA* (EH05) pZSKLMgldA was tested at high glycerol levels where it produced almost exclusively ethanol in minimum media at superior volumetric rates.

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1. Introduction

1.1 The biodiesel industry

Biofuels and biochemicals have attracted increasing interest as questions loom about the quantity of oil in the ground, the cost of extraction and the effect on air quality. In the past three years, oil prices have jumped 200% (Fig. 1) to a new all time inflation adjusted high due to political instability, increase in demand and a leveling off of supply, though recent prices are closer to historic levels (EIA 2009). Analysts at Goldman and Sachs stated oil could reach $200 a barrel due to supply and demand (The Star 2008). Prices have fallen in the last few months as the world has gone into a recession. However, prices will eventually raise again as there is only so much supply in the ground. The heads of several major oil companies are now publicly stating that we are close to peak oil, the time when the total barrels of oil per day plateaus (Guardian 2008). This inability to increase production becomes even more likely with political limitations imposed by many oil producing nations (Guardian 2008). This has led many countries, including the United States, to research domestic carbon sources.

A nation can cultivate various crops to produce biobased fuels and chemicals. This creates the opportunity for entrepreneurs to provide renewable carbon sources with an increase in revenue to farmers and local

economies and a decrease in the country's trade deficit. The price of biochemicals and biofuels will continue to be linked to oil prices as long as fossil fuels are used in large quantities. However, the price of biobased sources should be much more stable than that of oil because oil prices fluctuate wildly due to perceived supply and demand issues rather than the actual cost of production as can be noticed over the last few years (Fig. 1). As the use of biofuels and biochemicals increases, countries will find it much harder to use oil as a political weapon as the cost of fuel is smoothed out by another fuel source (Du & Hayes 2008).

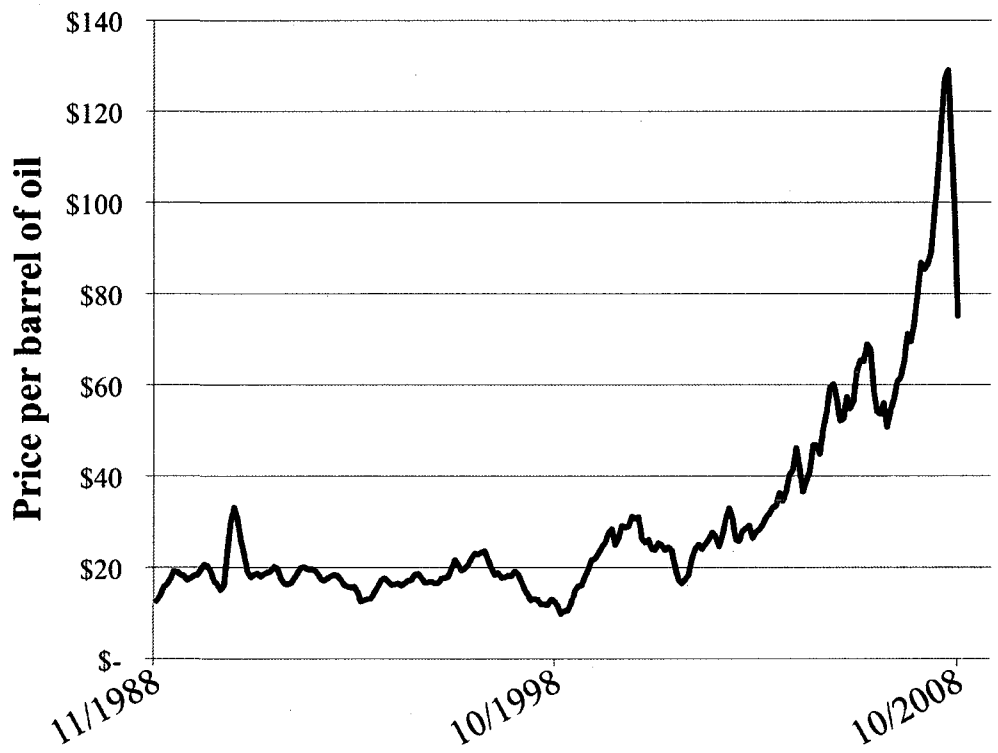


Figure 1. The monthly average refiner acquisition cost of crude oil in nominal dollars, not adjusted for inflation. (EIA 2009).

Biofuels burn cleaner than fossil fuel and thus produce less pollution. For example, biodiesel combustion generates a lower percentage of carbon monoxide, sulfur dioxide and particulate matter compared to diesel combustion; these byproducts cause health issues in large cities (Sheehan et. al. 1998). An equal amount of carbon dioxide, the primary cause of global warming, is released with the use of either fuel; however, biobased sources are a "closed loop system" - any carbon dioxide produced in their use was consumed when the biomass was grown, leading to a smaller increase in the Earth's temperatures (Hill et. al. 2006). Plant and animal biomass can be used as feedstocks to produce transportation fuels, synthetic gases and other chemicals through the use of chemical or biological processes (Henstra et. al. 2007, Marchetti et. al. 2007, Service 2007). The carbon footprint from utilizing biobased sources would be much less than the one from our current fossil fuel sources. If the world could replace fossil fuel use with the efficient use of biomass, humans and the world's environment would be much healthier.

More than 60% of the oil used in the United States goes to fuel its transportation system as gasoline and diesel (EIA 2008). These fuels can be replaced with ethanol and biodiesel, respectively, and considerable research has been done to create a multibillion dollar carbon industry. Currently,

ethanol is biologically fermented from sugars primarily from corn, sugarcane and sugarbeets while biodiesel is manufactured in a chemical transesterification process combining vegetable oils or animal fats with an alcohol (Gray et. al. 2006, Marchetti et. al. 2007).

However, current technology is unable to meet the challenge of increasing demands on biofuels (Odling-Smee 2007). In 2007, President Bush called for the United States to replace 20% of then current gasoline consumption, 35 billion gallons a year, with ethanol by 2017 (Service 2007). Using corn to achieve this goal would have serious repercussions; Searchinger found that if farmers converted 43% of current US grain cropland to growing corn for ethanol, it would produce less than 15 billion gallons of ethanol (Searchinger 2008). Nevertheless, there are several promising developments that might provide a better process. Among them are the utilization of cellulose (a mostly indigestible carbohydrate that forms the insoluble walls of cells in plant mass) to generate ethanol, and the use of engineered algae to grow quickly with a high oil content that can easily be converted into biodiesel (Schubert 2006, Xu et. al. 2006). These approaches have the potential to expand biobased carbon sources beyond prime farmland in addition to making the process more environmentally friendly, and energy efficient (Schubert 2006, Xu et. al. 2006).

The market ultimately drives all production, and both current and future production must be economically viable. Biodiesel created from soybean, the dominant feedstock, has very low profitability. While last year's oil prices hit an all time high, at times, biodiesel margins were negative due to the expensive soybean feedstock. As of the beginning of 2009, soybean biodiesel cost $2.98 a gallon to produce, and was sold for about $3.15 leaving only a 5% profit margin (Jacobson). This margin was higher previously; partly due to the byproduct soap crude which is 80% glycerol at cost $0.25/lb in 2004 (Sheehan et. al. 1998, Jacobson 2009). However, since then the market price has fallen as low as $0.02/lb due to a glycerol glut (Fig. 2), and is currently priced at $0.055/lb (Jacobson 2009). Biodiesel production creates about 1 pound of soap crude per gallon; furthermore, US facilities have had a 10-fold increase in biodiesel in the last three years (Fig. 2) as production grew from less than 30 million gallons in 2004 to a capacity of more than 2 billion at the end of 2007 (McCoy 2005, Biodiesel 2008). This dramatic growth has created more glycerol than the market could bear, and most synthetic glycerol makers (who sold to food, nitroglycerin and toothpaste manufacturers) have shut down, including the glycerol operations of Dow Chemical and Procter & Gamble Chemicals (McCoy 2005). Small biodiesel facilities will compost or burn the glycerol

rather than try to sell it. If there were more demand for glycerol, it would make the biodiesel industry more profitable and robust.

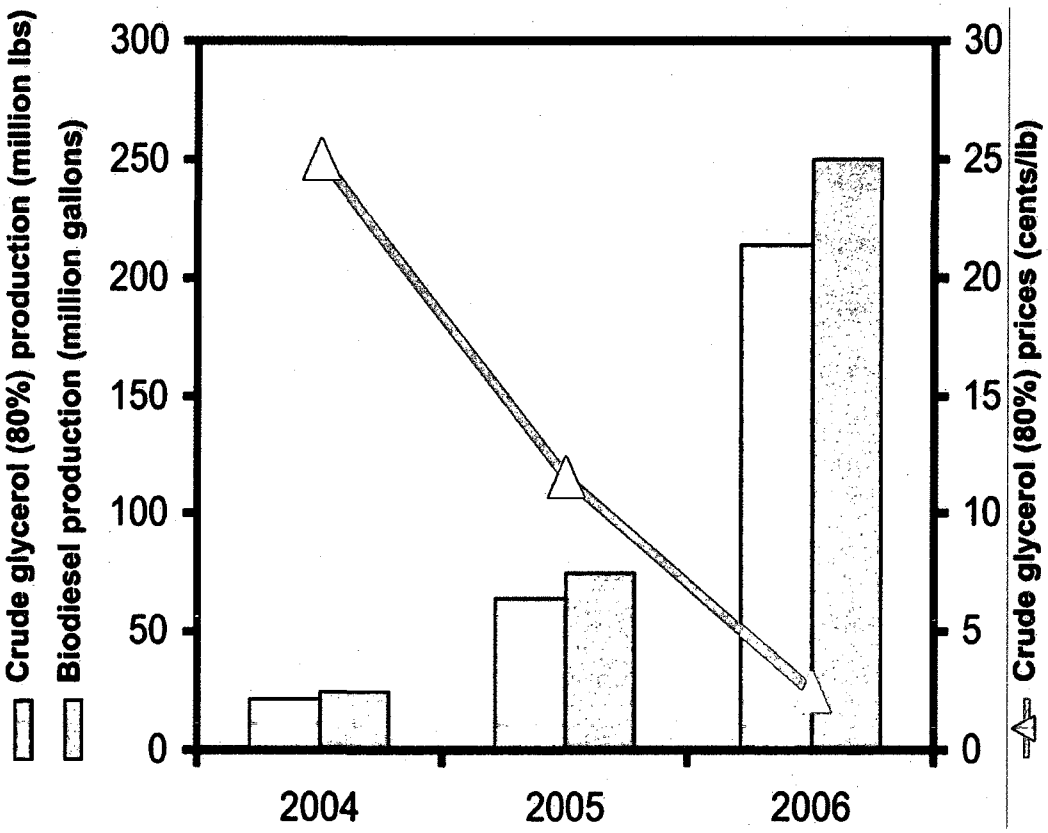


Figure 2. US biodiesel production and its impact on crude glycerol prices (Jacobson 2009). Figure originally appeared in Yazdani 2007.

1.2 The advantages of glycerol fermentation

Instead of burning glycerol, it can instead be converted into more valuable chemicals. Biodiesel facilities potentially could setup biorefineries that would utilize the waste glycerol as a feedstock and increase the profitability of the entire enterprise (Kamm & Kamm 2007). Stand-alone

businesses could purchase the glycerol streams from companies in either the biodiesel or oleochemical industries if the glycerol producing organizations didn't want to diversify (Kamm & Kamm 2007, Hazimah et. al. 2003). Glycerol's current prices are comparable to other feedstocks such as glucose. While glycerol can be a reactant in a chemical process, it requires high temperature and pressure conditions. Conversely, bacterial processes do not require such temperatures and pressures, and can utilize the cheaper source of soap crude without it necessarily being purified (Ito 2005). While several biochemical industries use other carbon sources such as glucose, glycerol has the advantage of being more reduced; the average degree of reduction per carbon, κ, of glycerol (equal to 4.7) is greater than that of either glucose or xylose ($\kappa_{glucose/xylose}$=4) (Nielsen et. al. 2003). In fact, glycerol produces twice as much reducing equivalents per kilogram when converted to phosphoenolpyruvate (PEP) as compared to glucose or xylose as shown in Figure 3 (Nielsen et. al. 2003). When a substrate is oxidized, all reducing equivalents produced must be consumed in the production of reduced products so a pathway is reduction-oxidation (redox) balanced (Nielsen et. al. 2003). As glycerol is more reduced than glucose, less carbon is wasted in undesirable products and instead can be utilized for highly reduced products. A biological process could theoretically convert 1 kg of glucose into 1 kg of

succinate, while a similar mass of glycerol could yield 1.28 kg of succinate if there was an external CO_2 source (Dharmadi et. al. 2006). Similarly, while 1 kg of glucose could theoretically produce about the same amount of ethanol as a kg of glycerol (about ½ kilogram of ethanol), the glycerol to ethanol pathway would synthesize 0.5 kg of formate in addition (Dharmadi et. al. 2006). The carbon in ethanol is more reduced than in glucose, and so a cell has to waste some of the reducing equivalents of this carbon for the pathway to be redox balanced (Dharmadi et. al. 2006). However, the glycerol-ethanol pathway is redox balanced, so nothing is wasted (Nielsen et. al 2003). Later in this report, we will demonstrate that microaerobic conditions can best take advantage of the redox-balanced pathways.

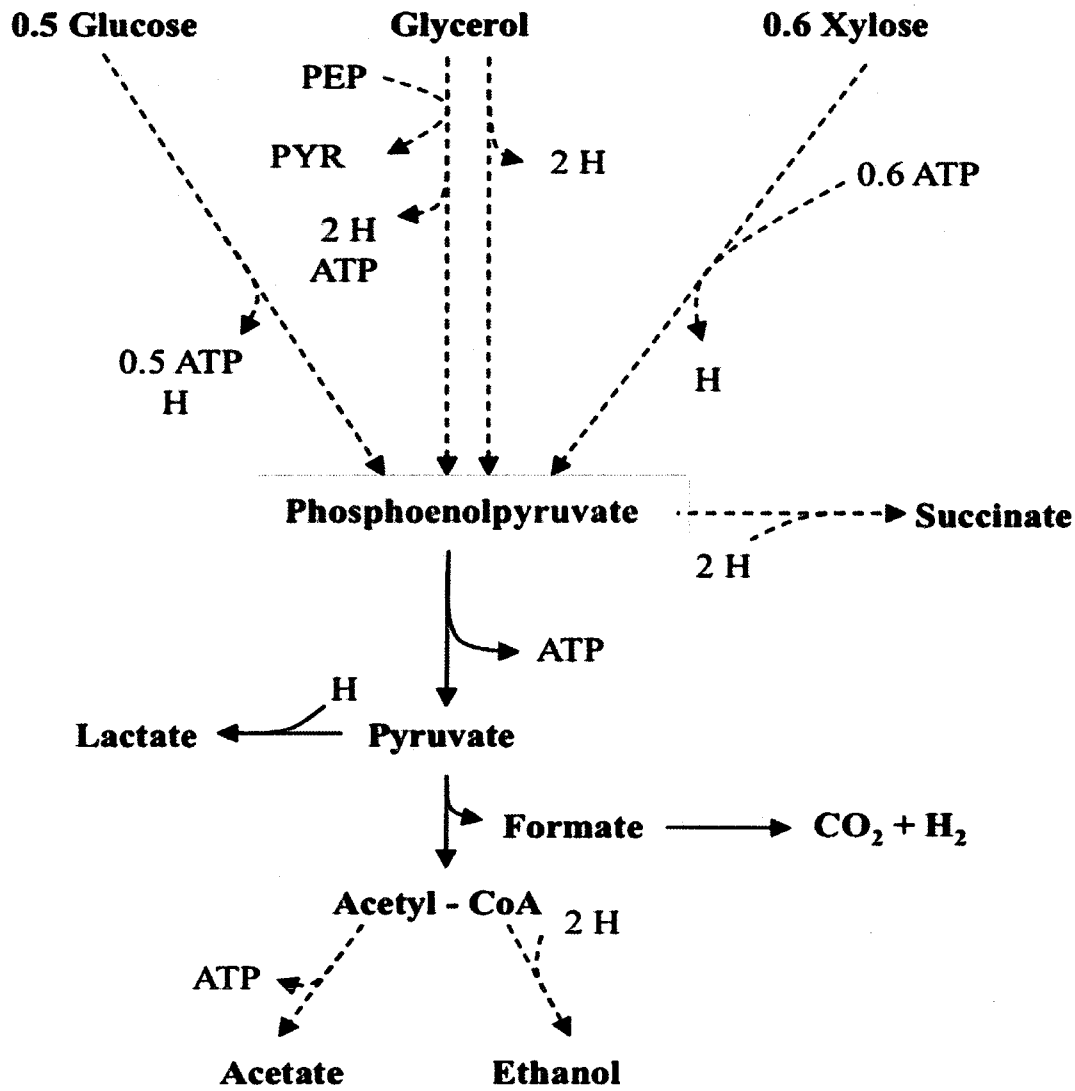


Figure 3. Generation and consumption of ATP and reducing equivalents during the conversion of glycerol, glucose and xylose to glycolytic intermediates and metabolic products. Broken lines illustrate multiple steps. H, NADH/FADH; PEP, phosphoenolpyruvate; PYR, pyruvate.

1.3 Glycerol fermentation by various strains

Glycerol can be utilized by a large number of microorganisms; however, due to glycerol's highly reduced state, most organisms are unable to consume glycerol in anaerobic conditions and must instead utilize external electron

acceptors such as oxygen (Schuller 2003, Booth 2005). Nevertheless, there are several bacterial strains- *Citrobacter freundii* and *Klebsiella pneumoniae*, as well as some in the *Enterobacteriaceae* family- that can ferment glycerol by producing 1,3-propanediol (1,3-PDO) a highly reduced product with $\kappa_{1,3\text{-PDO}}$=5.3 (Bouvet et. al. 1995, Nielsen et. al. 2003). Cell mass is less reduced than glycerol ($\kappa_{1,3\text{-PDO}}$=4.3) and so the 1,3-PDO pathway is used for redox balance as it consumes 2 reducing equivalents (Bouvet et. al. 1995). Several other microorganisms in the *Clostridium family* use this pathway, including *C. butyricum*, *C. pasteurianum*, and some genetically engineered strains of *C. acetobutylicum* (Gonzalez-Pajuelo et. al. 2005). While other strains can anaerobically ferment glycerol, they require a secondary carbon source to do so. Both a *K. planticola* strain and an *E. aerogenes* mutant metabolize glycerol to ethanol when fed complex nutrients; however, *K. planticola* requires a very long fermentation time and both are potentially pathogenic (Jarvis et. al. 1997, Ito et. al. 2005). The *Propionibacteria* strains *P. acidipropionici* and *P. freudenreichii* produce propionic acid while *Anaerobiospirillum succiniciproducens* had high yields of succinate (Bories et. al. 2004, Lee et. al. 2001). Nevertheless, these strains, like almost all, are unable to utilize glycerol as a substrate without an additional carbon source. In addition, the strains listed have several other

traits that do not lend well to research. Some are dangerous, are strict anaerobes or just are not studied enough to be used for genetic modification due to a dearth of the right genetic tools or the actual knowledge of their microbial pathways. As mentioned, the studies performed generally utilize complex nutrients to augment the glycerol carbon source, as these strains are not developed enough to do without.

1.4 Glycerol fermentation by E. coli

Escherichia coli is an organism that is extremely well studied and understood. It is adaptable and very readily accepts genetic modifications, making it an excellent model for genetic engineering. Previous studies have engineered *E. coli* strains for sugar fermentation to make biochemicals (Gonzalez 2005). Most strains are non-pathogenic and they can live in aerobic or microaerobic environments. However, it was long believed that wild-type *E. coli* was unable to subsist on glycerol as a substrate without external electron acceptors and so the only attempts to use *E. coli* for glycerol fermentation was through the addition of 1,3-PDO producing genes from other species (Zhu et. al. 2002, Booth 2005, Zhang et. al. 2006). Nevertheless, our lab recently discovered that *E. coli* could in fact anaerobically ferment glycerol with an addition of relatively low amounts of

tryptone (Dharmadi et. al. 2006, Yazdani & Gonzalez 2007, Murarka et. al. 2008). *E. coli* appears to be a poor choice for glycerol fermentation due to the inability of the wild-type to anaerobically ferment glycerol without complex nutrients. However, *E. coli* can easily be genetically engineered and evolved so the superior strains created are better than what is available using other organisms.

1.5 Microaerobic conditions enable cheaper fermentation of glycerol

Although our research group has genetically modified *E. coli* to better ferment glycerol and produce reduced products such as ethanol, it is still an expensive and time-consuming process (Yazdani & Gonzalez 2007, Yazdani & Gonzalez et. al. 2008). The ability to ferment in microaerobic conditions could alleviate both of these issues, as *E. coli* could utilize oxygen as an external electron acceptor and be able to grow on glycerol more rapidly without the need for an additional carbon source. However, care must be taken to balance the requirements of growth with carbon flux to reduced products. Too much oxygen would initially up-regulate cell growth and production of lesser-valued metabolic products such as acetate at the expense of reduced products (Losen et. al. 2004). Too little oxygen would prevent sufficient cell growth. As the cell mass grows, the microorganisms

have less oxygen available per cell and are instead forced to produce reduced products with the carbon instead. As the glycerol- ethanol pathway is redox balanced and has a net energy balance, once there is ample cell mass to quickly produce reduced products, the available oxygen is only used for regenerating the cell mass. Fine-tuning the oxygen supply will allow for both quick glycerol consumption and high reduced product yield.

1.6 Hypothesis

We hypothesized a minimal oxygen influx (through the use of air in the headspace), will allow *E. coli* to ferment glycerol without the need of an additional carbon source while still maintaining a high carbon flux recovery in reduced products. In addition, we hypothesized removal of several respiratory genes (*aceF*, *glpK*, *glpD*) should prevent growth in microaerobic conditions, while the deletion of several seeming inessential genes required for synthesis of metabolic by products (*fdhF, frdA, poxB*) would not affect glycerol consumption. Lastly, the strains SY03 pZSKLMgldA and SY04 pZSKLMgldA would have an equal or greater volumetric ethanol yield in microaerobic conditions than when grown anaerobically with rich nutrients.

1.7 Objectives

The aim of this study is to continue the research in the use of *E. coli* to produce reduced products from glycerol. Prior studies have discussed cell growth in anaerobic conditions to better understand the mechanisms of cells lacking external electron acceptors. This study focuses on how microaerobic conditions can be utilized to best synthesize reduced products such as ethanol from glycerol in the cheapest manner possible.

Specific objectives are described below and will be discussed in detail later:

- Establish microaerobic conditions that best enable the conversion of glycerol to reduced products using minimal medium.
- Identify metabolic pathways mediating/enabling the utilization of glycerol under microaerobic conditions.
- Demonstrate the feasibility of converting glycerol to ethanol and co-products formate and hydrogen.

2. Glycerol fermentation- a review

2.1 Introduction on glycerol fermentation

Very few microorganisms can ferment glycerol without external electron acceptors or a secondary carbon source because glycerol is so highly reduced. A total of only eight taxa of *Enterobacteriaceae* (as well as a number of other families of microorganisms) are reported to consume glycerol this way, and they must utilize the reductive 1,3-PDO pathway to balance the oxidative biomass pathway (Bouvet et. al. 1995). Bouvet used *Klebsiella pneumoniae* as a model of how this pathway allows for growth on glycerol. The coenzyme B_{12}-dependent glycerol dehydratase (encoded by gene *glyD*) dehydrates glycerol to 3-hydroxypropionaldehyde (3-HPA). Reducing equivalents produced when glycerol is oxidized to cell mass are consumed as 1,3-PDO dehydrogenase (1,3-PDODH) utilizes them to produce 1,3-PDO from 3-HPA (Booth 2005). Until very recently, this was believed to be the only way glycerol could be anaerobically fermented without a secondary carbon source.

2.2 Glycerol fermentation by E. coli

It was previously believed that *E. coli* could only consume glycerol either through the use of external electron acceptors or the heterologous expression of 1,3-PDO pathway genes since wild-type *E. coli* lacks *glyD* and therefore cannot produce the enzyme 1,3-PDODH (Bouvet et. al. 1995, Zhu et. al. 2002, Booth 2005, Zhang et. al. 2006). However, our research group recently discovered that different *E. coli* strains are able to ferment glycerol with the appropriate medium composition due to the 1,2 PDO pathway found in the wild-type shown in Figure 4 (Booth 2005, Dharmadi et. al. 2006, Yazdani & Gonzalez 2007, Murarka 2008). Growth of any microorganism in a fermentative environment requires both redox balance and ATP generation. Wild-type *E. coli* utilizes the redox-balanced glycerol to ethanol pathway for energy production. However, because cell mass is less reduced than glycerol ($\kappa_{cell\ mass}$=4.3, κ_{glyero}=4.7) the pathway is not redox balanced and would lead to a buildup in reduction equivalents (Nielsen et. al. 2003). However, the complex nutrients in the media are sufficient for some cell growth, and there is also minimal production of 1,2-PDO; this metabolite has a similar reduction degree of carbon as 1,3-PDO with $\kappa_{1,2\text{-}PDO}$=5.3 (Fig. 4) and allows redox balance with biosynthetic pathways (Murarka et. al. 2008, Nielsen et. al. 2003).

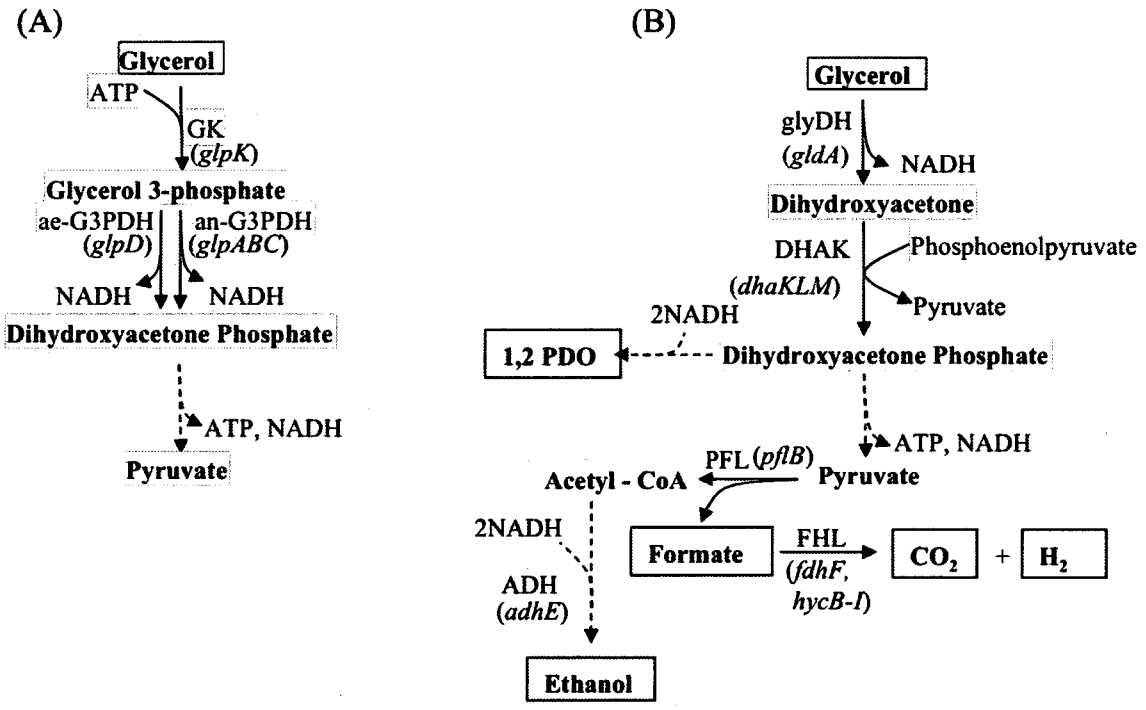


Figure 4. Pathways involved in the respiratory and fermentative utilization of glycerol in *E. coli*. A: Glycerol dissimilation in the presence of electron acceptors (i.e., respiratory metabolism) is mediated by an ATP-dependent glycerol kinase and two respiratory glycerol-3-phosphate dehydrogenases. B: Glycerol utilization in the absence of electron acceptors (i.e., fermentative metabolism) is mediated by a type II glycerol dehydrogenase and a phosphoenolpyruvate-dependent dihydroxyacetone kinase. Relevant enzymes and genes are shown. Broken lines illustrate multiple steps. ADH, acetaldehyde/alcohol dehydrogenase; ae-G3PDH, aerobic glycerol-3-phosphate dehydrogenase; an-G3PDH, anaerobic glycerol-3-phosphate dehydrogenase; DHAK, dihydroxyacetone kinase; FHL, formate hydrogen-lyase; GK, glycerol kinase; glyDH, glycerol dehydrogenase; PFL, pyruvate formate-lyase; QH, reduced quinones; 1,2 PDO, 1,2-propanediol.

2.3 Metabolic pathways mediating the conversion of glycerol to glycolytic intermediates

Glycerol is transported into the cell primarily through the glycerol diffusion protein GlpF (coded by *glpF*), as well as by osmosis (Heller et. al. 1980, Tobimatsu et. al. 1999). Once inside the cell, glycerol may be metabolized to dihydroxyacetone phosphate (DHAP) by two different

pathways- GldA-DHAK and the GlpK-GlpD/GlpABC, with GlpK-GlpD primary (Booth 2005, Yazdani & Gonzalez 2007, Gonzalez et. al. 2008, Murarka et. al. 2008). The enzyme glycerol kinase (GK, coded by *glpK*) metabolizes glycerol to glycerol-3-phosphate (G3P) (Booth 2005). It has been proposed that enzymes GlpK and GlpF are physically associated with one another, as they are both encoded by the operon *glpFKX*, making it easier for GK to phosphorylate glycerol. This process consumes phosphate in the form of one ATP (Thorner 1975). One potential reason for GlpK-GlpD being the primary pathway may be glycerol transportation is highly dependent on GK activity (Zwaig et. al. 1970). The enzyme GlpT (coded by *glpT*) can transport G3P into and out of the cell by allowing inorganic phosphate to flow in the opposite direction to prevent a toxic buildup of either molecule (Huang et. al. 2003). An anaerobic or aerobic pathway can oxidize G3P to DHAP in a reaction that produces 2 reducing equivalents. Aerobic G3P dehydrogenase (ae-G3PDH, coded by *glpD*) can use nitrate or oxygen to oxidize G3P and is required in aerobic conditions (Schryvers et. al. 1978, Austin & Larson 1991). In a fermentative environment, the enzyme anaerobic G3P dehydrogenase (an-G3PDH, coded by *GlpABC*) oxidizes the majority of G3P to DHAP. Originally, it was thought an-G3PDH required the exterior electron acceptor fumarate, and without

fumarate *E. coli* would metabolize glycerol to G3P and subsequently be unable to metabolize the G3P and thus could not survive (Zhu et. al. 2002, Booth 2005). However, as our research group discovered, once the glycerol is metabolized to DHAP, it can be further metabolized to 1,2-PDO in a process that consumes two reducing equivalents (Murarka et. al. 2008). In addition to the primary pathway between glycerol and DHAP, there is a secondary one that first oxidizes glycerol to dihydroxyacetone (DHA). The enzyme glycerol dehydrogenase (GldA, encoded by *gldA*) produces two reducing equivalents in this fermentative process (Paulsen et. al. 2000). Dihydroxyacetone kinase (DHAK, coded by *DhaKLM*) then metabolizes DHA to DHAP utilizing the phosphate in phosphoenolpyruvate (PEP), which is later metabolized to pyruvate (Jin & Lin 1984). Cells directly synthesize methylglyoxal (MG) from DHAP, and can indirectly synthesize cell mass if necessary (Sedivy et. al. 1984, Donahue et. al. 2000, Booth 2005).

When there is excess glycerol inside the cell, the imbalance between glycerol uptake and the flux through glycolysis can be corrected by producing MG. When cells are phosphate limited, they will produce MG in response. In both cases, DHAP is dephosphorylated to MG by methylglyoxal synthase (MGS, coded by *mgsA*) (Booth 2005). Cells

generally only produce MG when either glycerol or phosphate levels are too high and too low, respectively, since MG is a toxin that inhibits cell growth and *E. coli* uses it to correct the substrate balance and re-enable cell growth (Booth 2005). However, this feedback loop can accumulate enough MG to kill the cells if the amounts of glycerol and its immediate products continue to accumulate. Once cells have stable levels of substrate, they can detoxify the MG by metabolizing it to 1,2-PDO. This allows the cell the ability to correct an imbalance in flux in between the upper and lower parts of glycolysis while also possibly producing the pathway for a reducing equivalent sink in the use of 1,2-PDO (Ferguson 1999).

Cells utilize DHAP as the building block for biomass production. DHAP and G3P are combined to form fructose-1,6-bisphosphate (F-1,6-B) via the enzymes fructose bisphosphate aldolase class I and II, encoded on *fbaB* and *fbaA* respectively. When the substrate is glucose, bisphosphate aldolase I is more active, while bisphosphate aldolase II predominates in the presence off 3-carbon molecule feed sources (Stribling & Perham 1973). *E. coli* utilizes two different fructose 1,6-bisphosphatases (FBPase), FBPase I (*fbp*) and FBPase II (*glpX*), to produce fructose-6-phosphate (F6P) from F-1,6-B. F6P can then enter the pentose phosphate chain to produce cell mass (Fraenkel 1965, Sedivy et. al. 1984, Donahue et. al. 2000). FBPase I is

required by *E. coli* for growth on glycerol in aerobic conditions and was thought to be the only FBPase. An anaerobic version of the enzyme called FBPase II was recently discovered, but inactivation of FBPase does not entirely prevent cell growth. Plasmid copies of FBPase II can overcome the lack of FBPase I in aerobic conditions (Donahue et. al. 2000).

2.4 Role of pathways involved in the synthesis of metabolic products originating from phosphoenolpyruvate, pyruvate, and acetyl-CoA

When wild-type *E. coli* is not in completely aerobic conditions (where glycerol is consumed to produce solely cell mass, carbon dioxide and water), it can synthesize several products depending on the availability of external electron acceptors. The overall pathway must be balanced between the reducing and oxidizing equivalents and it must produce energy. Cells can synthesize acetate, ethanol, formate, hydrogen, lactate and succinate in this process (Guest 1992). All of these products are derived from pyruvate except for succinate, which originates from PEP. Again, redox balance is required; if the cell is unable to use all reducing equivalents in product production that it synthesizes in substrate consumption, it will be forced to stop metabolizing carbon. In addition, the cell must also have a net production of energy to be able to maintain cell mass and to grow. These

requirements are the two limiting steps that determine what the cells produce so they can thrive in their environment.

When external electron acceptors are in abundance, cells will only produce CO_2, H_2O and cell mass. As less and less oxygen is available, cells are unable to produce only oxidized products and must instead begin producing acetate as well as the reduced products ethanol, lactate and succinate to have redox balance when growing on glycerol (Fig. 3) (Sawers & Clark 2004). When no external electron acceptors are available, the cell will produce predominately ethanol, since the glycerol to ethanol pathway is redox balanced. Cell mass and any potential acetate are most likely synthesized from the secondary carbon source (Murarka et. al. 2008). The product mix is determined by the reduction state of the product and the amount of external electron acceptors (Sawers & Clark 2004). The wild-type *E. coli* requires the production of both ethanol and acetate to survive in fermentative conditions on either glucose or glycerol substrates; however, succinate and lactate synthesis are not required (Sawers & Clark 2004, Dharmadi et. al. 2006, Mat-Jan et. al. 1989, Murarka et. al. 2008). Interestingly, the double mutant *Δpta ΔadhE* prevents both ethanol and acetate production and will grow on glucose anaerobically because the

glucose-lactate pathway is redox balanced (Gupta & Clark 1989, Sawers & Clark 2004).

E. coli has three possible fermentation pathways for a slightly reduced substrate like glucose. Cells can either produce lactate, ethanol and acetate, or succinate. Lactate will form at low pH in mostly anaerobic conditions (Mat-Jan et. al. 1989, Sawers & Clark 2004). Predominant succinate production occurs in situations with only substrates less reduced than glucose or with rich media as a secondary carbon source (Donnelly et. al. 1998, Chatterjee et. al. 2001, Sawers & Clark 2004). *E. coli* synthesizes relatively little succinate because the amount of co-produced energy is much less than that of other products (Fig. 3). Any energy generated in the PEP to succinate pathway is by the proton motive force (PMF, mentioned below) and is less than one ATP (Lawford & Haddock 1973, Gennis & Stewart 1996). Cells require energy, generally in the form of ATP, to sustain cell growth and viability. ATP is used to maintain the gradients across the plasma membrane, produce several metabolites required for cell survival and help transport several necessary molecules inside the cell, as well as allowing others (like RNA) to be degraded and regenerated on a continuous basis (Murarka 2008). Succinate synthesis includes little energy production with glycerol fermentation (the metabolism of glycerol to PEP has zero net

energy), so most carbon flux flows from PEP to pyruvate generating one ATP.

Regardless of energy production, some carbon flux must flow from PEP to succinate as oxaloacetate (OAA) is part of this pathway and is an essential metabolite for cell growth. Phosphoenolpyruvate carboxylase (Ppc, coded on *ppc*) consumes one H_2O and one CO_2 molecule as it dephosphorylates PEP to OAA, a four carbon molecule. This CO_2 can be generated in the pyruvate to Acetyl-CoA pathways; insufficient CO_2 levels limit the amount of succinate that can be produced (Sawers & Clark 2004). This can be alleviated either through the addition of bicarbonate directly to the media or by sparging CO_2 gas (Murarka et. al. 2008). Inactivation of Ppc prevents production of OAA; without this precursor metabolite *E. coli* is unable to grow in either anaerobic or microaerobic conditions (Neidhardt et. al. 1996). OAA is reduced to malate by malate dehydrogenase (*mdh)* which is dehydrated to fumarate by fumarase A, B or C (coded by *fumA, fumB* and *fumC*, respectively) (Cronan & LaPorte 2006, Bell et. al. 1989, Guest 1992, Guest & Russell 1992, Woods & Guest 1987). The fumarate is then further reduced to succinate by fumarate reductase (coded by *frdABCD*) in a reaction that creates energy through the proton motive force (PMF). The energy from PMF is the sum total created when the cells produce succinate

from glycerol and is less than the energy in one ATP (Sawers & Clark 2004, Gennis & Stewart 1996, Ingledew & Poole 1984). While the glycerol to succinate pathway is redox balanced, so is the glycerol to ethanol pathway, which also will produce 1 full ATP: this means little succinate is synthesized (Sawers & Clark 2004).

As mentioned, one ATP is produced when pyruvate kinase I and II (coded by *pykA and pykF*, respectively) dephosphorylate PEP to make pyruvate. The pyruvate can be reduced to lactate or have a carbon cleaved through either the pyruvate oxidase (PoxB) pathway, or the Acetyl-CoA producing pathways of pyruvate dehydrogenase (PDH) and pyruvate formate-lyase (PFL); these enzymes are coded by *poxB*, *aceEF* and *lpdA*, and *pflB*, respectively. The lactate pathway is least optimal - it produces no additional energy and it only consumes two reducing equivalents, preventing redox balance in glycerol fermentation. Lactate can be synthesized with a less reduced carbon as a substrate or with external electron acceptors. Carbon flows through the PoxB, PDH or PFL pathways dependent on the availability of reducing equivalents, which is reflected in the NADH/NAD^+ ratio.

In favorable redox balance conditions, pyruvate is reduced to lactate via D-Lactate dehydrogenase (D-LDH, coded by *ldhA*). This pathway is not

integral to either glycerol or glucose fermentations; wild-type *E. coli* grown on glycerol do not produce any lactate, while *ΔldhA* mutants grown on glucose have carbon consumption rates similar to that of the wild-type (Mat-Jan et. al. 1989, Sawers & Clark 2004, Dharmadi et. al. 2006). More aerobic conditions enable cells to produce some lactate as the excess reducing equivalents from glycerol are consumed by respiration (Guest and Russell 1992, Sawers & Clark 2004). Several studies have shown that lactate production can occur in anaerobic batch reactors in the later part of the fermentation once acidic products have lowered the pH. Interestingly, studies have shown that the system must be both anaerobic and have a lower pH for high transcription of *ldhA* (Sawers & Clark 2004, Mat-Jan et. al. 1989, Clark 1989). Nevertheless, D-LDH is not strictly anaerobic, as it still active in aerobic conditions (Sawers & Clark 2004). In addition, the strain *E. coli B* will mostly produce lactate in glucose fermentation at neutral pH (Lawford & Haddock 1998). D-LDH appears to be activated by high levels of pyruvate, which can occur when carbon is not flowing through the PFL pathway (Neidhardt et. al. 1996). Lactate production allows cells to be redox balanced when consuming glucose; however, due to glycerol's highly reduced state, *E. coli* is unable to metabolize glycerol to lactate in fermentative conditions. It has been found that lactate synthesis is more

likely to occur later in the fermentation when conditions are more acidic and anaerobic (Clark 1989, Mat-Jan et. al. 1989). It is postulated this helps the cells deal with acid stress from the organic acids already produced (Neidhardt 1996).

The pathways PoxB, PDH and PFL metabolize most of the carbon flux in glycerol fermentation. The amount of external electron acceptors and the reduced state of the substrate determine the pathway; PDH is deactivated with relatively low levels of oxygen, while PFL is deactivated when there are few to none external electron acceptors. When the $NADH/NAD^+$ ratio is very close to one, PoxB is most active (Guest & Russell 1992, Gennis & Stewart 1996, Cassey et. al. 1998).

In general, pyruvate will flow through the PDH or PFL pathways. However, PoxB can act as an alternative by oxidizing and decarboxylating some pyruvate to acetate and CO_2 while producing energy and a reducing equivalent (Gennis & Hager 1976, Bertagnolli & Hager 1991, Guest & Russell 1992, Bertagnolli & Hager 1993, Abdel-Hamid et. al. 2001). This pathway is not essential for growth and produces less energy than is possible when acetate is produced via the PDH or PFL pathways (Fig. 3). It has been suggested that PoxB is mostly active during microaerobic conditions, PFL is mostly inactive due to too much oxygen and there is too few electron

acceptors for PDH to be the primary enzyme. Studies have demonstrated that *poxB* expression is most active in the early stationary phase and is important in the transition between exponential and stationary growth (Guest & Russell 1992, Gennis & Stewart 1996). It allows mutants lacking the PDH pathway to grow very slowly in aerobic conditions (Gennis & Hager 1976).

Pyruvate is mostly metabolized by the PDH and PFL pathways, this is dependent on the NADH/NAD^+ ratio (Guest & Russell 1992, Chang et. al. 1994). The PDH pathway is important for growth in aerobic conditions; however, it is not required, while mutants lacking PFL cannot grow anaerobically (Varenne et. al. 1975, Langley & Guest 1977, Mat-Jan et. al. 1989, Kaiser & Sawers 1994, De Graef et. al. 1999, Sawers & Clark 2004). Nevertheless, it has been demonstrated that PDH flux does occur in anaerobic environments while limited PFL flux can occur in microaerobic environments (Tseng et. al. 1996). PFL is the central pathway in fermentation the cells are able to cleave AcCoA into formate and pyruvate without exterior electron acceptors, while the PDH pathway oxides AcCoA and produces two reducing equivalents and one CO_2 (Kessler & Knappe 1996, Knappe & Sawers 1990, Sawers & Watson1998, Sawers 1999).

Two enzymes regulate PFL activity. PFL activase (coded by *act*) activates PFL by adding a free radical when high NADH/ NAD^+ ratios exist and PFL deactivase (coded by *adhE*) deactivates PFL when the ratio is low. PFL deactivase is very sensitive to oxygen and will quickly deactivate PFL. Active PFL is unstable in aerobic conditions, exhibiting a half-life of 10 seconds at 0°C (Sawers & Clark 2004). Nevertheless, flux through PFL still occurs in microaerobic conditions where the oxygen saturation is at 1% or lower. In fact, when the dissolved oxygen levels are at 0.5 to 1%, both respiration and fermentation occur because PDH and PFL are both active (Sawers & Clark 2004).

The reducing equivalents produced when PFL decarboxylates pyruvate are utilized in the synthesis of formate. At a non-acidic pH, formate will build up in the media. At an acidic pH with no additional external electron acceptors, formate will be further reduced to H_2 and CO_2 by the enzyme formate hydrogen-lyase (FHL) (Stephenson & Stickland 1931, Stephenson & Stickland 1932, Sawers et. al. 2004). FHL activity requires two separate enzymes- formate dehydrogenase (FDH, coded by *fdhF*) and hydrogenase (Hyd coded by the hyc operon)- as well as two electron acceptors (Gest & Peck 1955, Peck 1957, Sawers et. al. 2004). The CO_2 produced can be used in the production of succinate from PEP, while

the hydrogen gas must escape the fermentation media for cell growth to continue. When hydrogen gas is trapped in the media, reducing equivalents build up and cell growth stops (Dharmadi et. al. 2006).

AcCoA can either be reduced to ethanol or dephosphorylated depending on the reduction state of the substrate and the existence of external electron acceptors. If more reducing equivalents must be consumed, ethanol is produced; otherwise acetate and ATP are synthesized (Dawes & Foster 1956, Clark & Cronan 1980 I, Clark & Cronan 1980 II, Singh et. al. 2006). *E. coli* will produce as much energy as possible from each molecule of glycerol dependent on the redox balance; with enough external electron acceptors they will just produce ATP, CO_2 and H_2O. With a limited amount of oxygen available, the cells will have redox-balanced pathways that produce as much acetate as possible. Acetate is the most oxidized product after carbon dioxide with a total of two ATP produced via the glycerol- PDH pathway. Almost no acetate is produced in the anaerobic glycerol fermentation because reduction equivalents would accumulate. However, in mostly aerobic conditions, a significant amount of acetate is synthesized from glycerol (Sawers & Clark 2004, Dharmadi et. al. 2006, Murarka et al. 2008, Gennis & Stewart 1996). The enzyme acetyl-CoA-orthophosphate acetyl transferase (PTA, coded by *pta*) is required for the

first step in producing acetate from AcCoA. PTA metabolizes pyruvate to acetyl phosphate and CoA (Sawers & Clark 2004). Then ATP-acetate phosphotransferase (ACK, coded by *ackA* and *ackB*) synthesizes acetate and ATP from the acetyl phosphate and an ADP. As mentioned, this ATP is an extra energy molecule that cannot be co-produced with any other fermentative product. In fact, *E. coli* will still utilize this pathway in a completely aerobic environment when the central metabolic pathways (such as the TCA/citric acid cycle) are unable to consume all the carbon (Kumari et. al. 1995). When the AcCoA to acetate pathway is blocked, it partially inhibits the PFL pathway (Singh et. al. 2006).

When there are too many reduction equivalents, cells will use them to synthesize ethanol from AcCoA. This pathway is necessary for wild-type fermentation of both glucose and glycerol. As mentioned, the double mutant Δ*pta*Δ*adhE* can ferment glucose because the glucose- lactate pathway is redox balanced (Gupta & Clark 1989, Sawers & Clark 2004). However, glycerol is more reduced, so because it cannot synthesize ethanol, the strain the strain is unable to survive without external electron acceptors (Dharmadi et. al. 2006, Murarka et. al. 2008). Most carbon flux in a glycerol fermentation flows through the ethanol pathway for redox balance. The AdhE enzyme (coded by *adhE*) first reduces acetyl-CoA to acetaldehyde and

then further reduces this metabolite to ethanol; in total the process consumes 4 reduction equivalents (Rudolph et. al. 1968, Cunningham & Clark 1986, Goodlove et. al. 1989, Sawers & Clark 2004). As with PFL, AdhE is positively affected by the $NADH/NAD^+$ ratio. The ratio is higher when there are no external electron acceptors; in this case, redox balance requires synthesis ethanol. With either exterior electron acceptors or a less reduced substrate, cells produce more acetate and less ethanol (Leonardo et. al. 1993). When the $NADH/NAD^+$ ratio is too low, the AdhE enzyme deactivates PFL (Kessler et. al. 1991, Kessler et. al. 1992).

3. Materials and methods

3.1. Strains, plasmids, and genetic methods

Strain BW25113, a derivative of the F-, λ-, *E. coli* K-12 strain BD792 (Datsenko & Wanner 2000), along with its otherwise isogenic single-gene knock out derivatives were obtained from the National BioResource Project (NIG, Japan) (Baba et. al. 2006). For simplicity, these single-gene knockout mutants are referred to throughout the paper using the following nomenclature: *Δgene_name*, where "*gene_name*" is the disrupted gene. Wild-type K12 *E. coli* strain MG1655 was obtained from the University of Wisconsin *E. coli* Genome Project and used as the host to implement metabolic engineering strategies (www.genome.wisc.edu, Kang et. al. 2004). Single gene knock out mutants from NIG were used as donors of specific mutations (Baba et. al. 2006). Gene knockouts were introduced in MG1655 and its derivatives by P1 phage transduction as described elsewhere (Miller 1972). All strains, along with primers and plasmids used in their construction, are listed in Table 1.

Table 1. Strains, plasmids and primers used in this study.

Strain/ Plasmid/Primer	Genotype/Structure/Description		Source
Strains			
MG1655	F- λ- *ilvG- rfb*-50 *rph*-1		Kang et. al. 2004
BW25113	$lacI^q$ $rrnB_{T14}$ Δ$lacZ_{WJ16}$ *hsdR*514 Δ*araBA-D*$_{AH33}$ Δ*rhaBAD*$_{LD78}$		Baba et. al. 2006
Δ*gldA*	BW25113; Δ*gldA-FRT-Km-FRT*		Baba et. al. 2006
Δ*dhaK*	BW25113; Δ*dhaK-FRT-Km-FRT*		Baba et. al. 2006
Δ*glpD*	BW25113; Δ*glpD-FRT-Km-FRT*		Baba et. al. 2006
Δ*glpK*	BW25113; Δ*glpK-FRT-Km-FRT*		Baba et. al. 2006
Δ*glpA*	BW25113; Δ*glpA-FRT-Km-FRT*		Baba et. al. 2006
Δ*frdA*	BW25113; Δ*frdA-FRT-Km-FRT*		Baba et. al. 2006
Δ*pta*	BW25113; Δ*pta-FRT-Km-FRT*		Baba et. al. 2006
Δ*ldhA*	BW25113; Δ*ldhA-FRT-Km-FRT*		Baba et. al. 2006
Δ*adhE*	BW25113; Δ*adhE-FRT-Km-FRT*		Baba et. al. 2006
Δ*pflB*	BW25113; Δ*pflB-FRT-Km-FRT*		Baba et. al. 2006
Δ*aceF*	BW25113; Δ*aceF-FRT-Km-FRT*		Baba et. al. 2006
Δ*fdhF*	BW25113; Δ*fdhF-FRT-Km-FRT*		Baba et. al. 2006
Δ*poxB*	BW25113; Δ*poxB-FRT-Km-FRT*		Baba et. al. 2006
SY03	MG1655, Δ*frdA*::*FRT* Δ*pta-FRT-Km-FRT*		Yazdani & Gonzalez 2008
SY04	MG1655, Δ*fdhF*::*FRT* Δ*frdA*::*FRT* Δ*pta-FRT-Km-FRT*		Yazdani & Gonzalez 2008
EH03	MG1655, Δ*frdA*::*FRT* Δ*pta*::*FRT*		Durnin et. al. 2009
EF04	MG1655, Δ*fdhF*::*FRT* Δ*frdA*::*FRT* Δ*pta*::*FRT*		Durnin et. al. 2009
EH05	MG1655, Δ*frdA*::*FRT* Δ*pta*::*FRT* Δ*ldhA-FRT-Km-FRT*		Durnin et. al. 2009
EF06	MG1655, Δ*fdhF*::*FRT* Δ*frdA*::*FRT* Δ*pta*::*FRT* Δ*ldhA-FRT-Km-FRT*		Durnin et. al. 2009
Plasmids			
pCP20	$rep_{pSC101}{}^{ts}$ Ap^R Cm^R *cI*857 l P_R flp+		Datsenko & Wanner 2000
pZSKLMGldA	*E. coli dhaKLM* and *gldA* genes under control of $P_{LtetO-1}$ (tetR, oriR SC101*, cat)		Yazdani & Gonzalez 2008
Primers[a]			
ldhA	cgtaatatcagggaatgaccc	gggcaaagaatgtcaaaaacaa	Durnin et. al. 2009

[a] Primers (5' to 3': forward sequence following reverse sequence) were used for verification of the *ldhA* mutation transferred from strain Δ*ldhA* to strains EH03 and EF04 via P1 phage transduction.

Various dilutions of P1 phage were mixed with the donor cells at exponential growth phase and tryptone top agar (1% tryptone, 0.5% NaCl, 0.65% Agar, 5 mM $CaCl_2$) and overlaid on Luria-Bertani broth (LB) agar plates containing 5mM $CaCl_2$ (Yazdani 2008). Plates showing many isolated plaques were used to collect phage lysates by adding 2 ml P1 adsorption media (LB + 5mM $CaCl_2$) and incubating overnight at 4°C (Yazdani 2008). The phage lysate was further shaken with chloroform to remove bacterial cell background and used to re-infect the donor cells to enrich for the mutation (Yazdani & Gonzalez 2008). The enriched phages were mixed with the recipient strain at a multiplicity of infection of 0.5 and incubated in 1XA media (10.5 g/L K_2HPO_4, 4.5 g/L K_2HPO_4, 1 g/L $(NH_4)2SO_4$ and 0.5 g/L sodium citrate-$2H_2O$) for 2 hours (Yazdani & Gonzalez 2008). Cells were centrifuged and plated on LB plates supplemented with appropriate antibiotics. The plates were incubated overnight at room temperature and then shifted to 30°C, and further grown for 1-2 days until colonies appeared. Colonies were purified on fresh LB plates, incubated at 42°C to cure the remaining lysogens, and further grown at 37°C (Yazdani & Gonzalez 2008). To eliminate the kanamycin resistance marker, the mutants were transformed with pCP20, a temperature sensitive plasmid expressing flippase (FLP) recombinase (Datsenko & Wanner 2000).

FLP expressed from this plasmid removed the Km region from the FRT::Km::FRT site, leaving one FRT site behind. pCP20 was then removed by growing the cells at 43°C. All mutations created in the host cells were confirmed by polymerase chain reaction using "verification" primers (Yazdani & Gonzalez 2008). The disruption of multiple genes in a common host was achieved by sequentially implementing the procedure described above. This sequential procedure, in turn, could lead to chromosomal rearrangements resulting from FLP-promoted recombination events between FRT sites at different loci (Datsenko & Wanner 2000). To verify that these events did not occur in strains with multiple gene disruptions, each strain was rechecked in all disruption sites by PCR at every step where a new mutation was introduced (Yazdani & Gonzalez 2008).

The construction of plasmids used in this study is illustrated elsewhere (Yazdani & Gonzalez 2008). The control plasmid pZSblank was constructed by digesting pZSKLcf with *Kpn*I and *Bam*HI restriction endonucleases, blunting overhangs with T4 DNA polymerase, and ligating the vector backbone with T4 DNA ligase (Bachler et. al. 2005). The expression vector pZSgldA was constructed as follows. The coding region of the *gldA* gene was PCR amplified using genomic DNA of *E. coli* MG1655 as template and "c1-gldA" primers. The restriction enzyme sites

*Kpn*I and *Pst*I were introduced through the forward and reverse primers, respectively, to facilitate cloning of the PCR product in the expression vector pZSKLM (Bachler et. al. 2005). The amplified product (1.1 kB) was digested with *Kpn*I and *Pst*I and used for ligation at the corresponding sites of the pZSKLM plasmid. The expression vector pZSKLMGldA was constructed by amplifying the *gldA* gene along with its ribosome binding site from the genomic DNA of *E. coli* MG1655 using "c2-gldA" primers (Yazdani & Gonzalez 2008). The amplified product was digested with *Pst*I and *Mlu*I and cloned at the corresponding sites of pZSKLM, downstream of the *dhaKLM* gene. The PCR was performed using Pfu turbo DNA polymerase under standard conditions described by the supplier (Stratagene, CA, USA). The ligated products described above were used to transform *E. coli* DH5 T1 (Invitrogen, CA, USA). Positive clones were screened by plasmid isolation and restriction digestion.

Standard recombinant DNA procedures were used for gene cloning, plasmid isolation and electroporation. Manufacturer protocols and standard methods (Miller 1972, Sambrook et. al. 1989) were followed for DNA purification (Qiagen, CA, USA), restriction endonuclease digestion (New England Biolabs, MA, USA) and DNA amplification (Stratagene, CA, USA and Invitrogen, CA, USA). The strains were kept in 32.5% glycerol stocks

at -80°C. Plates were prepared using Luria-Bertani (LB) medium containing 1.5% agar, and appropriate antibiotics were included at the following concentrations: 34 mg/ml chloramphenicol and 50 mg/ml kanamycin.

3.2. Culture medium and cultivation conditions

The minimal medium (MOPS) designed by Neidhardt et. al. (1974) with 1.32 mM Na_2HPO_4 in place of K_2HPO_4 and supplemented with 20 g/L glycerol, 1 mM sodium selenite, 3.96 mM Na_2HPO_4, 5 mM $(NH_4)_2SO_4$, and 30 mM NH_4Cl was used unless otherwise specified. Chemicals were obtained from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich Co. (St Louis, MO).

Fermentations were conducted in an in house multi-fermentation system with six 300-ml working volume vessels (CGS-The Kontes Custom Glass Shop, Vineland, NJ). Each vessel was fitted with a double-tube condenser maintained at 4°C by using a Fisher Scientific Isotemp Refrigerating Circulator 30165 (Thermo Fisher Scientific, Waltham, MA). Temperature, pH, and agitation were controlled as follows. The vessels were immersed in a water bath, whose temperature was maintained at 37.5°C with a Lauda E-100 series immersion circulator (Brinkmann, Westbury, NY). This allowed the fermenter temperature to be maintained at 37°C. The pH

was controlled at either 6.3 or 7.5 with a Jenco 3671 pH Controller fitted with a Jenco 600p pH probe (Jenco, San Diego, CA). 2 M NaOH base for pH control was added by gravity flow using a Pinch Valve (Bio-Chem Inc., Boonton, NJ) connected to the pH controller. The stirrer speed was maintained at 300 r.p.m by using a Lab-Line Multi-Magnestir Model 1278 (Thermo Fisher Scientific, Waltham, MA). Microaerobic conditions were established by flushing zero gas (standard mix) air through the headspace of the fermenters at a flow rate of 0.02 liter per min. The dissolved oxygen concentration was measured with a DO-BTA dissolved oxygen sensor (Vernier Software & Technology, Beaverton, OR). We confirmed the proper oxygen flow rate through the media by making sure the DO concentration leveled off near 6.5 mg/L after about 30 minutes starting from a 0 mg/L concentration. Operation under these conditions resulted in decreasing dissolved oxygen concentrations that fell below the detection limits after 2 hours of cultivation (i.e., undetectable dissolved oxygen concentrations during almost the entire course of the fermentation).

Prior to use, the cultures (stored as glycerol stocks at -80°C) were streaked onto LB plates and incubated overnight at 37°C. Ten colonies were used to inoculate 250 ml Pyrex bottles (Thermo Fisher Scientific, Waltham, MA) containing 175 ml of minimal medium supplemented with 10 g/L of

glycerol. The bottles were incubated at 37°C and 150 r.p.m. in a NBS C24 Benchtop Incubator Shaker (New Brunswick Scientific Co., Inc., Edison, NJ) until an OD_{550} of ~0.3 was reached. An appropriate volume of this actively growing pre-culture was centrifuged, and the pellet was washed and used to inoculate 300 mL of medium in each fermenter, with a target initial optical density at 550 nm of 0.05. In some experiments, specified in each case, the minimal medium for the inoculum phase was supplemented with 10 g/L tryptone and 5 g/L yeast extract and/or the initial optical density in the fermenters was set at OD_{550} of 0.50.

3.3. Analytical methods

Optical density was measured at 550 nm and used as an estimate of cell mass (1 OD_{550} = 0.34 g dry weight/L). After centrifugation, the supernatant was stored at -20°C for HPLC (High Performance Liquid Chromatography) and NMR (Nuclear Magnetic Resonance) analysis. Glycerol, organic acids, ethanol, and hydrogen were quantified in an ion-exclusion HPLC using a Shimadzu Prominence SIL 20 system (Shimadzu Scientific Instruments, Inc., Coumbia, MD) equipped with an HPX 87H organic acid column (Bio-Rad, Hercules, CA) as described in Dharmadi (2005). The operating conditions consisted of a 30 mM H_2SO_4 inmobile

phase with deionized water, column temperature 42°C and flow rate of 0.3 mL/min. The transfer of oxygen in microaerobic cultures was characterized by calculating the volumetric oxygen transfer rate (N_{O2} in mg O_2/L/h) as $N_{O_2} = k_L a\left(C^* - C_L\right)$. k_L is the oxygen transport coefficient (cm/h), a is the gas-liquid interfacial area (cm^2/cm^3), $k_L a$ is the volumetric oxygen transfer coefficient (h^{-1}), C^* is the saturated dissolved oxygen concentration (mg/L), and C_L is the actual dissolved oxygen concentration in the liquid (mg/L) (Shuler 2002). The volumetric oxygen transfer coefficient ($k_L a$) was estimated by the static gassing out method (Shuler 2002). In these calculations, C^* was assumed to be 6.5 mg/L. During microaerobic operation, the dissolved oxygen concentration was essentially zero and therefore the volumetric rate of oxygen uptake equals the volumetric rate of oxygen transfer and can be calculated as $N_{O_2} = k_L a \times C^*$ (N_{O2} in mg O_2/L/h). The average specific rate of consumption of oxygen (mg O_2/g cell/h) was then obtained by dividing N_{O2} by the time-average concentration of cells. The first two hours of the fermentation had measurable amounts of oxygen and are referred to as aerobic conditions.

3.4. NMR Experiments

The identity of the fermentation products was determined through NMR experiments. Sixty µl D_2O and 1 µl of 600 mM NMR internal standard TSP (3-(Trimethylsilyl) propionic acid-D4, sodium salt) were added to 540 µl of the sample (culture supernatant). The resulting solution was then transferred to a 5 mm-NMR tube and 1D proton NMR spectroscopy was performed at 25°C in a Varian 500MHz Inova spectrometer equipped with a Penta probe (Varian, Inc., Palo Alto, CA). The following parameters were used: 8,000 Hz sweep width; 2.8 s acquisition time; 256 acquisitions; 6.3 µs pulse width; 1.2 s pulse repetition delay; and presaturation for 2 s. The resulting spectrum was analyzed using FELIX 2001 software (Accelrys Software Inc., Burlington, MA). Peaks were identified by their chemical shifts and J-coupling values, which were obtained in separate experiments where samples were spiked with metabolite standards (2 mM final concentration).

3.5. Enzyme activities

Cells from microaerobic cultures were harvested by centrifugation (3 min, 10,000 × g), washed twice with 9 g/liter NaCl, and stored as cell pellets at -20°C. Cell pellets were resuspended in 0.1 M potassium phosphate

buffer to obtain ~3.4 mg dry cell weight/ml, and permeabilized with chloroform (Murarka et. al. 2008, Tao et. al. 2001, Osman et. al. 1987). The assay for measuring anaerobic glycerol-3-phosphate dehydrogenase (G3PDH) activity is a modification of the assay described by Kistler & Lin (1972), using the following final concentrations: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 75 μM; phenazine methosulfate, 600 μM; DL-glycerol 3-phosphate, 10 mM; Triton X-100, 0.2%; flavine adenine dinucleotide (FAD), 10 μM; and flavine mononucleotide (FMN), 1 mM. The aerobic G3PDH activity was determined in the same way, except that flavines were omitted and sodium cyanide (10 μM) was included in the assay. The assays were monitored spectrophotometrically at 570 nm. The extinction coefficient of reduced MTT was 17 $mM^{-1}cm^{-1}$. Glycerol kinase activity was assayed as described by Kistler (1972), by measuring the change in absorbance at 340 nm and 25 °C in a 1 mL reaction mixture containing 0.15 M glycine, 11 mM $MgCl_2$, 0.27 M hydrazine, 1.2 mM NAD^+, 5 mM ATP, 2 mM glycerol, 20 U of a-glycerophosphate dehydrogenase, and 50 mL crude cell extract prepared as described above.

The activity of alcohol dehydrogenase was measured as described by Kessler et. al. (1991). Cells pellets, harvested as described above, were

resuspended in 0.2 mL of 0.1M MOPS-KOH and permeabilized by vortex mixing with chloroform. The activity was assayed by measuring the change in absorbance at 340 nm and 30°C in a 1 mL reaction mixture containing 0.4 mM NADH, 10 mM acetadehyde or 0.2 mM acetyl-CoA and 50 mL crude cell extract.

The activity of glycerol dehydrogenase towards the oxidation of glycerol was measured as reported by Truniger & Boos (1994). Cells pellets prepared as described above were resuspended in 0.2 ml of potassium carbonate buffer (pH 9.5) and permeabilized by vortex mixing with chloroform. The activity was assayed by measuring the change in absorbance at 340 nm and 25°C in a 1 ml reaction mixture containing 2 mM $MgCl_2$, 500 μM NAD^+, 100 mM glycerol, 100 mM of potassium carbonate buffer (pH 9.5), and 30 μl crude cell extract (Truniger & Boos 1994). Dihydroxyacetone kinase activity was assayed using the method reported by Kornberg & Reeves (1972) with minor modifications. Cells from microaerobic cultures (OD_{550} of ~0.7) were harvested by centrifugation (3 min, 10,000 × g), washed with decryptification buffer (0.1 M-sodium-potassium phosphate, pH 7.2, and 5 mM $MgCl_2$) and stored as cell pellets at -20°C (Durnin et. al. 2009). The pellets were resuspended in decryptification buffer to obtain ~1 mg dry cell weight/ml. A portion of the

ice-cold cell suspension was placed in a test tube, vigorously mixed for one minute, and 0.2 volume of toluene-ethanol (1:9, v/v) added. The assay was conducted in a 1 ml reaction mixture containing 1 mM phosphoenolpyruvate trisodium, 0.1 mM NADH, 2U lactate dehydrogenase, and 50 mg dry weight of toluene-ethanol treated cells. The assay mixture was incubated at 30°C for 15 min. Dihydroxyacetone was added to a concentration of 1 mM and the decrease in absorbance was followed at 340 nm.

Linearity of the reactions (protein concentration and time) was established for all preparations. All spectrophotometric measurements were conducted in a BioMate 5 spectrophotometer (Thermo Scientific, MA, USA). The nonenzymatic rates were subtracted from the observed initial reaction rates. Enzyme activities are reported as mmol of substrate/minute/mg of cell protein and represent averages for at least three cell preparations.

3.6. Calculation of fermentation parameters

Data for cell growth, glycerol consumption, and product synthesis were used to calculate volumetric rates/productivities (mmol/L/h). Specific rates (mmol/g cell/h) were calculated by dividing the volumetric rates by the time-average concentration of cells. Growth and product yields (mol or

gram of product per mol or gram of substrate consumed) were calculated as the amount of cell mass or product synthesized per amount of glycerol consumed once the cultures reached the stationary phase, unless otherwise stated. The "oxygen yield", defined as the amount of oxygen consumed in the metabolism of a given carbon source, was calculated by dividing the volumetric rate of oxygen consumption by the volumetric rate of substrate consumption during the active growth phase. In the above calculations, we used an average molecular weight of 24.7, which corresponds to an average cell of a molecular formula $CH_{1.9}O_{0.5}N_{0.2}$ (Nielsen et. al. 2003). Several experiments demonstrated that the condenser was not enough to prevent some ethanol from evaporating and that a time based correction factor was required. All fermentation data has been corrected for the 0.0877% /h evaporation rate. Hydrogen production was calculated by contrasting the molar amount of formate produced in EF04 and EF06 (87%) to that of ethanol and acetate, and then assuming that this ratio will be very similar for most other strains. This assumption is based on the limited amount of carbon flux through both the POX and pyruvate dehydrogenase pathways. The analytical coefficient of variation is given for the data in tables and figures and is equal to the standard deviation/mean*100.

4. Results and Discussion

4.1 Microaerobic glycerol utilization and its metabolic products.

Previous studies by our group demonstrated that wild-type *E. coli* could survive on glycerol in fermentative conditions; however, they required an expensive supplement consisting of carbon rich nutrients that potentially makes the process cost prohibitive for commercialization (Dharmadi et. al. 2006, Yazdani & Gonzalez 2007, Murarka et. al 2008, Yazdani & Gonzalez 2008). As mentioned, cell growth in anaerobic conditions was dependent on a secondary carbon source due to glycerol's highly reduced state. However, with external electron acceptors in the media, the cells no longer required rich nutrients to produce cell mass (Durnin et. al. 2009). Low airflow across the fermentation media provided a relatively cheap way to add external electron acceptors. Under microaerobic conditions, a much larger percentage of the carbon was synthesized into cell mass instead of less reduced products; despite the shift, the production rate of high value products significantly increased (Durnin et. al. 2009).

The wild-type BW251113 anaerobically synthesized more than 97% of the carbon in glycerol to ethanol; however, less than 10 g/L of glycerol was fermented in 96 hours (Fig. 5A). Under proper microaerobic

conditions, the same strain had a ten-fold increase in glycerol consumption and maintained significant flux to reduced products (Durnin et. al. 2009).

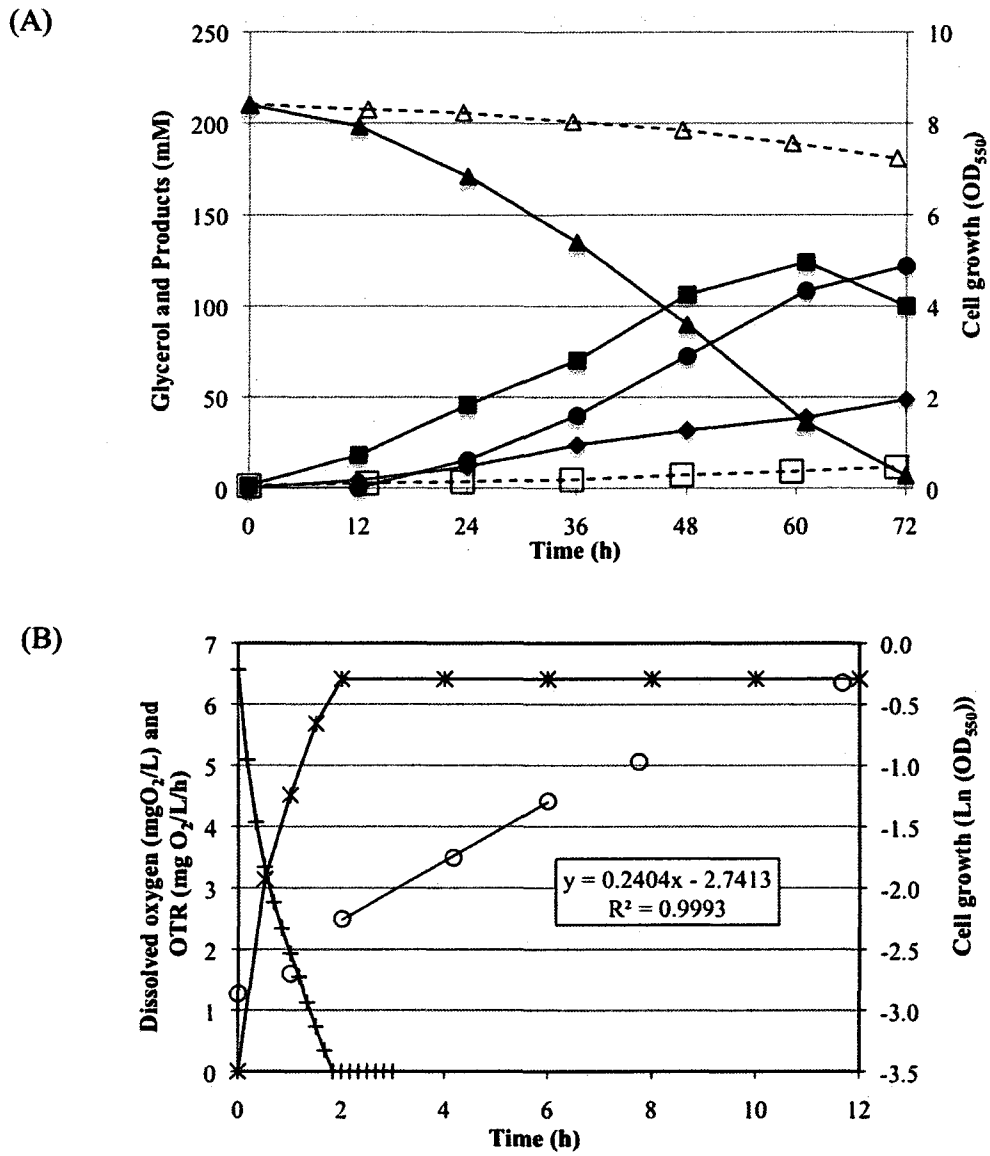


Figure 5. Glycerol utilization by *E. coli* BW25113 in a minimal medium under microaerobic conditions. **(A)** Concentration of cells (■), glycerol (▲), ethanol (●) and organic acids (◆). Cell density (□) and glycerol concentration (△) for the fermentative utilization of glycerol are also shown (broken lines: same mineral salts medium but supplemented with 2 g/L tryptone). **(B)** Dissolved oxygen concentration (+), volumetric rate of oxygen transfer (*), and log-linear plot of cell density (■) around the exponential growth phase. Fitting of the exponential-phase data to a straight-line model once microaerobic conditions are established is shown (least-squares method). Coefficient of variation was below 4.9% in all cases. Figure originally in Durnin et. al. 2009.

While similar results were found for other strains including MG1655, W3110, MC4110, and *E. coli B* etc. (data not shown), strain BW25113 was chosen for the initial research because our research group already had a full set of BW25113 mutants as well as knowledge of how these mutants grew under anaerobic conditions. After the background investigation was completed, this study performed fermentations on genetically engineered strains for ethanol production. All engineered strains were with a MG1655 background instead of wild-type BW25113, as BW25113 is not a true wild-type. It is actually a derivative of *E. coli* W3100, with several gene deletions that cause it to produce less succinate and grow slightly slower than MG1655 (Durnin et. al. 2009).

To determine optimum microaerobic conditions, the metrics high overall carbon recovery (both including and excluding biomass), high carbon recovery in reduced products, and volumetric rates were utilized. The optimum conditions were found at a mixing rate of about 300 RPM with airflow across the surface of the fermentation media equal to 0.067 VVM (Durnin et. al. 2009). These parameters allowed for a much greater cell growth rate without sacrificing a high percentage of carbon recovery or high rate reduced product synthesis (Fig. 5A).

The fermentations started with high dissolved oxygen (DO) levels, but as the biomass grew aerobically, the DO quickly decreased to immeasurable levels within two hours (Fig. 5B). Once the dissolved oxygen levels reached zero, the biomass received a steady state influx of oxygen at 0.1448 mg/L/min and cells grew exponentially at a maximum specific growth rate of 0.24 h^{-1} (Fig. 5B). We estimated this environment had the volumetric oxygen transfer coefficient value (k_La) of 2.5 h^{-1} (Durnin et. al. 2009).

In the first 12 hours of fermentation, carbon was primarily utilized for cell growth with only 36% recovered into products- primarily acetate (data not shown). Almost complete aerobic conditions existed in the first few hours of the fermentation; with an O_2/cell ratio of 1 mg O_2/L/minute/OD the biomass was able to grow exponentially with efficient energy production in the TCA cycle. After six hours of growth, this ratio fell to less than 0.4 mg - O_2/L/minute/OD and continued to fall to less than 0.14 in the first 24 hours (Durnin 2009). As this occurred, the NADH/NAD^+ ratio fell and the cells synthesized reduced products for redox balance. BW25113 primarily synthesized ethanol throughout the fermentation (Durnin et. al. 2009).

We further analyzed the fermentative broth once cells reached stationary phase by utilizing 1D proton NMR to identify the products. The spectrum of a 60-hour fermentation sample from a similar culture in Figure

5A is shown in Figure 6. The only products found in the extracellular medium were ethanol, succinate, acetate and formate (Durnin et. al. 2009). Interestingly, 1,2-propanediol, a highly reduced product found after anaerobic fermentation of glycerol was not found, while it was in the anaerobic fermentation (Dharmadi et. al. 2006). Otherwise, the two fermentations synthesized the same types of products (Dharmadi et. al. 2006, Yazdani & Gonzalez 2008, Murarka et. al. 2008, Durnin et. al. 2009).

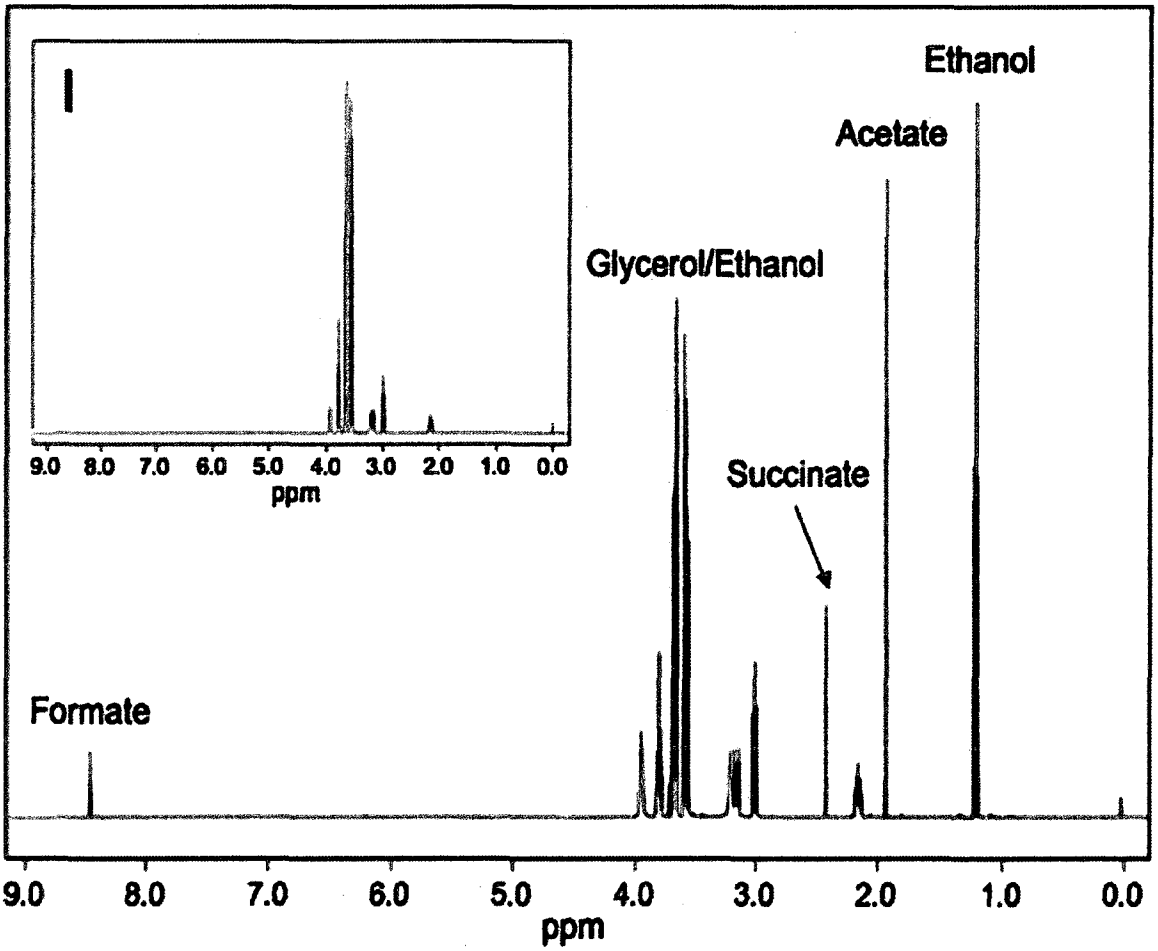


Figure 6. 1D 1H-NMR spectrum of the culture medium in a late fermentation sample (60 hours in a fermentation similar to that shown in Figure 5). The inset corresponds to the NMR spectrum of a time zero sample. Figure originally in Durnin et. al. 2009.

Table 2 shows the results for the microaerobic fermentation with about 90% of all carbon being recovered in products and the remaining 10% consumed for biomass synthesis with no carbon lost. The overall redox balance was also excellent, as there was less than a 7% discrepancy between the reducing equivalents generated and consumed (Table 2). As could be expected, most of the carbon flux and reducing equivalents were consumed in ethanol synthesis, 66% and 89%, respectively, although this is lower than in the anaerobic fermentation with 91% of carbon recovered into ethanol (Durnin et. al. 2009). There were relatively modest amounts of succinate and acetate, and most of the formate produced was synthesized to CO_2 and H_2 (Durnin et. al. 2009). Little glycerol was metabolized to succinate by BW25113 due to the gene deletions previously mentioned and the little energy co-produced with succinate (Durnin et. al. 2009). While both the ethanol and succinate pathways are redox-balanced, the ethanol pathway produces more energy. While formate was synthesized in large quantities as a co-product of ethanol, the enzyme formate hydrogen-lyase (FHL) converted formate to CO_2 and H_2 (Sawers et. al. 2004). If this fermentation had been conducted at a basic pH, the enzyme would be less active and more formate would have remained in the fermentative broth (Sawers et. al. 2004).

Table 2. Calculation of the fermentation balance for the growth of *E. coli* BW25113 on glycerol under microaerobic conditions at pH 6.3 and 37 °C. This sample was taken as the cells reached stationary phase; it relates to a 72-hour sample from the culture in Figure 5A. Table originally in Durnin et. al. 2009.

Substrate consumed and products formed	Moles of product/substrate per mole of glycerol[a]	Oxidation state[b]	Redox balance[c]	Carbon recovery (C atoms)[d]
Glycerol	1	- 2.0	-2.0	3.0
Ethanol	0.66	- 4.0	- 2.64	1.32
Acetate	0.18	0.0	0.00	0.37
Succinate	0.05	+ 2.0	+ 0.11	0.16
Formate	0.01	+ 2.0	+ 0.03	0.01
CO_2[e]	0.83	+4.0	+3.32	0.83
Hydrogen[e]	0.83	+2.0	+1.66	-
Cell mass[f]	0.10	- 0.3	- 0.03	0.10
Oxygen[g]	0.08	-2.0	-0.31	-
Sum[h]			+2.14	2.80

[a] Values are net moles produced per mole of glycerol fermented.
[b] The oxidation state of carbon atoms was calculated assuming oxidation states of -2 and +1 for oxygen and hydrogen, respectively (Sawers and Clark, 2004).
[c] Data in this column was obtained by multiplying the "moles of product per mole of glycerol" times the "oxidation state".
[d] Carbon recovery was calculated by multiplying the "moles of product per mole of glycerol" times the number of carbon atoms in the molecule.
[e] Moles of CO_2 or Hydrogen generated in the dissimilation of pyruvate were calculated assuming that moles of "CO_2 *plus* Formate" equals moles of "ethanol *plus* acetate".
[f] A molecular formula of $CH_{1.9}O_{0.5}N_{0.2}$ was assumed for an average *E. coli* cell (Nielsen et al., 2003).
[g] Moles of oxygen per mole of glycerol were calculated by dividing the volumetric rate of oxygen consumption by the volumetric rate of glycerol utilization during the active growth phase.
[h] The "sum" doesn't account for glycerol.

Recall that the major advantages of using microaerobic instead of anaerobic conditions were the savings both in cost and time. The media no longer required expensive complex nutrients, and glycerol consumption substantially increased. Anaerobic glycerol consumption was very slow because the cell mass grew only on the complex nutrients- in 72 hours the biomass had an OD_{550} of less than 0.5 (Durnin et. al. 2009). Meanwhile, the optical density of cells grown in microaerobic conditions was almost 5 OD_{550} This larger cell mass synthesized substantially more ethanol over the course of the fermentation at a rate of 5 g/L with volumetric reduced product rate of 1.90 mMol/L/h (Durnin et. al. 2009). In contrast, the anaerobic biomass produced almost entirely ethanol for the majority of the fermentation at 1.4 g/L and volumetric reduced product at 0.43 mMol/L/h (Durnin et. al. 2009). This four-fold increase in ethanol and succinate production coupled with the elimination of rich media made the microaerobic production of ethanol much cheaper than that of anaerobic.

4.2 Comparative analysis of glycerol, glucose and xylose utilization under microaerobic conditions.

Currently, commercial glycerol fermentation rarely occurs because it is generally cost prohibitive in comparison to the fermentation of less

reduced substrates such as glucose and xylose. In studies, *E. coli* consumed glucose seven times faster than glycerol without needing a secondary carbon source (Dharmadi et. al. 2006, Murarka et. al. 2008). This disparity was due to difference in degree of reduction per carbon, as $\kappa_{glycerol}$=4.7 and $\kappa_{glucose/xylose}$=4, especially in comparison to that of cell mass, assuming its molecular formula is $CH_{1.9}O_{0.5}N_{0.2}$, $\kappa_{cell\ mass}$=4.2 (Nielsen et. al. 2003). This means that the glucose and xylose pathway to cell mass consumes redox equivalents and can balanced with the energy producing acetate pathway. Cell mass subsisting on glycerol must maintain the redox balance by producing more of the less energy rich reduced products like ethanol and thus less of biomass. While this made it impossible for cells to anaerobically metabolize glycerol to cell mass, the higher reduction value of glycerol allowed for better recovery of highly reduced products as glycerol fermentation produced twice as many reducing equivalents as that of glucose or xylose (Fig. 3). With microaerobic conditions, the redox balance was maintained by reducing oxygen to water (Durnin et. al. 2009). However, most of the reducing equivalents were still available for the synthesis of highly reduced products, which meant the theoretical ethanol yield was higher from glycerol than that of glucose or xylose. The products synthesized from these three substrates were entirely dependent on the redox

balance and net energy balance and varied due to the differences between the carbon sources.

In the microaerobic environment specified, wild-type BW25113 consumed 20 g/L of glucose in less than 18 hours, 20 g/L of xylose in around 36 hours and 20 g/L of glycerol in less than 72 hours (data not shown). Glycerol consumption was still between two and four times slower than other substrates on a g/L basis; however, a much higher yield of high value products like ethanol were produced (Durnin et. al. 2009). Because glycerol is more highly reduced, it has a better oxygen yield, and *E. coli* grown on this substrate produced much more ethanol than acetate, giving an acetate/ethanol ratio of less than 0.277 (Durnin et. al. 2009). When BW25113 was grown microaerobically on glycerol, the ethanol yield was 66% (Durnin et. al. 2009). Conversely, on xylose or glucose the ethanol yield was much lower, 25% and 20%, respectively, because cells produced as little ethanol as possible to maintain redox balance (Fig. 7).

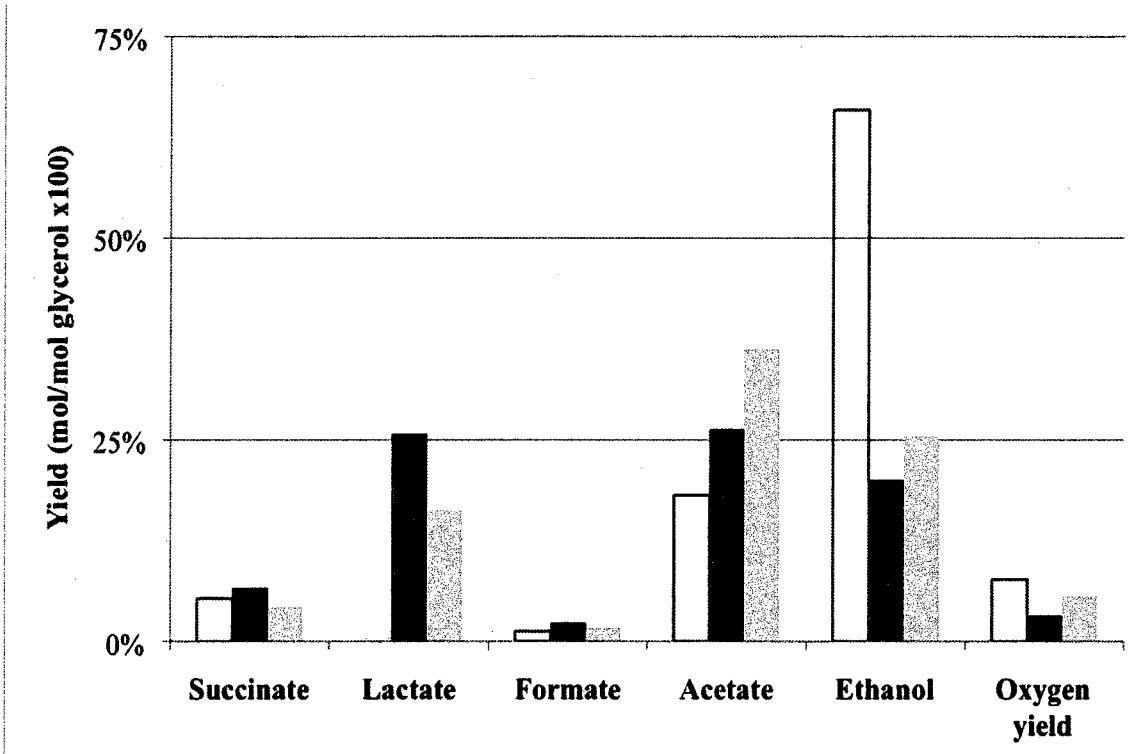


Figure 7. Product, biomass and oxygen yield during the microaerobic utilization of glycerol (white bars), glucose (black bars) and xylose (gray bars). The "oxygen yield" represents the amount of oxygen consumed in the metabolism of each substrate. Values represent the means from three samples taken in stationary phase BW25113 cultures. Coefficient of variation was below 4.3% in all cases. Figure originally in Durnin et. al. 2009.

Instead, the carbon flux in the glucose and xylose fermentations flowed more through the pathways to acetate and lactate because they are the highest energy producing pathways that are redox balanced. Xylose has less energy per pyruvate than the other two carbon sources (Fig. 3), so cells grown on this substrate produced a higher amount of acetate than when consuming glucose or glycerol (Fig. 7). The higher amount of acetate production required more electron acceptors for redox balance, which meant xylose metabolism had a higher oxygen demand than that of glucose (Durnin

et. al. 2009). Cells grown on glycerol had an even higher oxygen demand; however, this was due to the higher degree of reduction per carbon of glycerol.

The acetate/ethanol ratio for cells grown on xylose was 1.42 and 1.31 for glucose (Durnin et. al. 2009). Both values are higher than one, demonstrating a higher production of acetate over ethanol. Wild-type BW25113 did not produce any lactate on a glycerol substrate; however, biomass on both xylose and glucose produced significant quantities. This occurred because lactate is less reduced than ethanol; the glucose/xylose to lactate pathway is redox balanced while that of glycerol is not. About 16% of the carbon flux from xylose flowed to lactate, while almost 26% of carbon from glucose was incorporated into lactate (Fig. 7). This difference was again due to redox and energy balances; while both the lactate pathways from both substrates are redox balanced, the glucose pathway has a higher net energy value, while the xylose pathway produced less energy and so wasn't as satisfactory for cell growth. The difference in net energy production can be noticed in the growth curves, as the strains grown on glucose grow much quicker than those on xylose (data not shown).

Although cells have a very high conversion ratio of glycerol to ethanol, *E. coli* biomass grown on glucose or xylose metabolized the

substrate more quickly than on glycerol. Cells consumed glucose at a rate of 37.7 mmol/hr, xylose at 17.6 mmol/hr and glycerol at 2.6 mmol/hr. Ethanol was synthesized at a rate of 7.59 mmol/hr, 4.5 mmol/hr and 2.4 mmol/hr on glucose, xylose and glycerol, respectively (data not shown). In glucose and xylose fermentations, much more carbon was wasted growing biomass and less valued products compared to that of glycerol fermentations. However, the glucose and xylose fermentations still produced ethanol more quickly since the rate of substrate consumption was so much greater. While cell mass grew much quicker on glucose and xylose, the specific growth rates for cells grown on these two substrates were tenfold more than cells grown on glycerol. Cells had a specific growth rate of 44.3 mmol carbon consumed/hr/OD_{550} on glucose and 33.7 mmol/hr/OD_{550} on xylose, compared to 3.4 mmol carbon consumed /hr/OD_{550} on glycerol. Due to the high levels of growth and carbon wasted in low valued products, the specific ethanol rate for the glycerol fermentation compared much more favorably to that of glucose and xylose as the difference was only fourfold- 2.27 mmol/hr/OD_{550}, 8.9 mmol/hr/OD_{550} and 8.62 mmol/hr/OD_{550}, respectively. When the wild-type strain was grown on glycerol in anaerobic conditions, it produced much less ethanol than when glucose or xylose was the substrate (Dharmadi et. al. 2006). Microaerobic conditions led to much quicker

ethanol production on glycerol leading to a much smaller difference in ethanol production via the different substrates. While the wild-type still produced ethanol 4 times slower on glycerol, *E. coli* can be genetically engineered to metabolize glycerol more quickly as well as theoretically to metabolize all of the glycerol to ethanol. In comparison, the glucose/xylose to ethanol pathway is constrained by redox balance and could have a maximum carbon flux of only 50%. These genetically engineered strains would make ethanol production from glycerol in microaerobic conditions much more economically feasible. A recent paper by our research group demonstrated ethanol produced this way would be cheaper than current best practices (Yazdani & Gonzalez 2007).

4.3 Metabolic pathways mediating the conversion of glycerol to glycolytic intermediates.

While much has been published about how various genes and the enzymes they code for work, few papers explain microaerobic fermentation of glycerol, and none discuss *E. coli* in these conditions. *E. coli* used the reduction potential of oxygen, which removed the need for additional carbon sources and allowed the study to focus on just glycerol fermentation. This permitted additional fermentations to determine the required enzymes for

survival in either an aerobic or microaerobic environment. Better understanding of how these genes influence bacterial growth, glycerol consumption and product generation can be applied to genetic engineering strategies to produce an organism that optimally makes desired products.

As stated, this study used BW25113 to take advantage of data from previous work on its mutants under anaerobic conditions (Murarka et. al. 2008, data not shown). BW25113 produced less succinate than the MG1655 wild-type (Durnin et. al. 2009). We evaluated mutants with single gene deletions and compared them to the wild-type to help elucidate their metabolic pathways.

One of the most important areas in the metabolism of *E. coli* is the glycerol-DHAP diamond, the only entryway for glycerol into the phosphoenolpyruvate (PEP) metabolic pathways. Two different routes have been reported in the literature for the conversion of glycerol to dihydroxyacetone phosphate (DHAP) in *E. coli* (Fig. 8); each function under opposing respiratory conditions. The GlpK-GlpD/GlpABC route mostly works in a respiratory environment, while GldA-DHAK functions mostly in a fermentative environment (Yazdani & Gonzalez 2007, Gonzalez et. al. 2008, Murarka et. al. 2008).

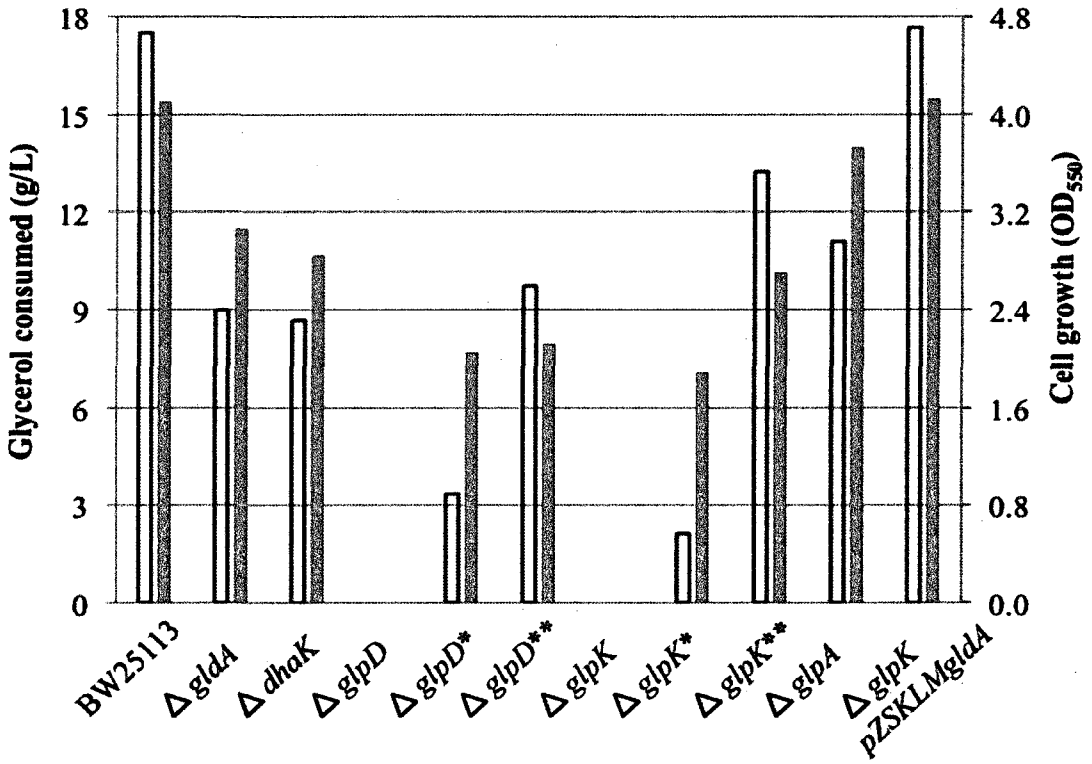


Figure 8. Identification of enzymes responsible for the microaerobic dissimilation of glycerol to glycolytic intermediate dihydroxyacetone-P (DHAP). Glycerol utilization (clear bars) and cell growth (gray bars) are shown. Unless otherwise specified, values represent the means for three samples taken at the earlier of: (i) the time at which a culture of the specific strain entered stationary phase or (ii) the time at which the BW25113 culture entered stationary phase. Coefficient of variation was below 6.5% in all cases. One asterisk (*) means the inoculum was grown in LB. Two asterisks (**) mean a high (10x) inoculum as well as being grown in LB. Figure originally in Durnin et. al. 2009.

As a beginning assessment, our group measured the corresponding enzymes when the cells were in their initial aerobic phase (with more than 50% saturation of dissolved oxygen) and in their microaerobic phase with no detectable oxygen concentrations (Durnin et. al. 2009). These time points were at one hour and 24 hours, respectively. All enzymes assayed had significant activity at both time points, demonstrating that both the

respiratory and fermentative pathways are active although each is predominant in different phases. The GlpK-GlpD pathway was most important initially due to significant dissolved oxygen levels per cell mass; however, the GldA-DHAK pathway became the dominant pathway once most of the dissolved oxygen was consumed (Table 3).

Table 3. Activities of enzymes involved in the conversion of glycerol to glycolytic intermediate dihydroxyacetone phosphate. Table originally in Durnin et. al. 2009.

Activity Measured[a]	Value ± standard deviation[a]	
	1-hour culture[b]	24-hour culture[b]
Glycerol kinase	0.167±0.002	0.01±0.05
Aerobic glycerol-3-phosphate dehydrogenase	0.028±0.006	0.029±0.002
Glycerol dehydrogenase	0.0038±0.0003	0.033±0.001
Dihydroxyacetone kinase	0.001±0.000	0.009±0.001

[a] All activities were measured as described in *Materials and Methods* and reported values are average ± standard deviation of triplicate assays. (Durnin et. al. 2009)
[b] Two BW25113 cultures were grown under microaerobic conditions as described in *Materials and Methods*. One culture was harvested after 1 hour, when the dissolved oxygen concentration was 50% of the saturation level. The second culture was harvested after 24 hours, when the dissolved oxygen concentration in the liquid was below detection limits.

This behavior correlates with data from single deletion strains (below) as well as with the proposed role of each of these pathways in the respiratory (GlpK-GlpD/GlpABC) and fermentative (GldA-DhaKLM) utilization of glycerol found by various research groups (Yazdani & Gonzalez 2007, Gonzalez et. al. 2008, Murarka et. al. 2008 and references therein). Taken together, this data demonstrates the importance of the fermentative pathway on cell mass production once oxygen levels become undetectable.

Fermentations were performed on strains with single gene deletions from the above pathways in the diamond (Δ*gldA*, Δ*dhaK*, Δ*glpK*, and Δ*glpD*). These mutants have characterized in previous studies, which showed they lack the enzyme activity (Gonzalez et. al. 2008, Murarka et. al. 2008). Remove of either the gene Δ*gldA* or Δ*dhaK* (which code for glycerol dehydrogenase and dihydroxyacetone kinase, respectively) significantly retarded cell growth and glycerol utilization by 30% and 50% for both mutant strains compared to the wild type BW25113 over the course of the fermentations (Fig. 8). While the data demonstrates that the GldA-DHAK pathway was not required for microaerobic growth on glycerol, it shows that it is still a significant carbon pathway. The Δ*gldA* and Δ*dhaK* fermentations had the same growth curves (data not shown) as well as similar glycerol consumption levels (Fig. 8). In fact, product composition was near identical, both metabolizing only 28% of glycerol to ethanol, compared to the BW25113 wild type strain at 66% (Durnin et. al. 2009). Additionally, the two mutants produced a much higher percentage of acetate- 43% than BW25113-16%, as they required additional energy for survival (Table 4).

While mutants lacking the GldA-DHAK pathway were able to grow in a microaerobic environment, initial research appeared to demonstrate the inactivation of either glycerol kinase (Δ*glpK*) or the aerobic glycerol

dehydrogenase (Δ*glpD*) prevented cell growth in the inoculum phase. Nevertheless, we believed that the hypothesis the GlpK-GlpD route was an absolute requirement for the microaerobic utilization of glycerol was incorrect. Significant activity of the alternative GldA-DHAK pathway in the wild type occurs, so it seemed unlikely that cells would not survive (Table 3). Additionally, the knockouts of *gldA* and *dhaK* negatively affected glycerol utilization and cell growth over the course of the entire experiment; this included the first 12 hours of the experiment with near aerobic conditions (Fig. 8, data not shown). If cells could only survive in a microaerobic environment with an intact GlpK-GlpD pathway, the Δ*gldA* and Δ*dhaK* mutants should have grown very similarly to the wild-type in the mostly aerobic initial conditions.

Table 4. Carbon recovery in cell mass and fermentation products during the microaerobic utilization of glycerol in minimal medium. Table originally in Durnin et. al. 2009.

Strain and culture conditions[a]	Carbon recovery[b]							
	Ethanol	Succinate	Acetate	Formate	Lactate	Biomass	Products	Overall
Wild-type strains								
BW25113	66	5	18	3	0	11	91	101
MG1655	52	13	16	3	0	10	85	95
Strains with gene deletions in glycerol utilization pathways								
Δ*gldA*	29	5	42	3	0	15	76	91
Δ*dhaK*	27	4	44	0	0	15	76	91
Δ*glpK***	76	5	12	6	0	10	93	103
Δ*glpD***	77	3	12	6	0	12	92	104
Δ*glpA*	56	3	19	6	0	13	78	92
Strains with gene deletions in pathways involved in the synthesis of metabolic products								
Δ*frdA*	67	1	19	3	0	11	87	99
Δ*aceF***	65	6	11	6	0	14	82	96
Δ*pflB*	3	1	8	0	67	7	79	86
Δ*poxB*	62	4	21	3	0	11	87	97
Δ*fdhF*	42	4	28	39	3	13	78	90
Δ*pta*	7	3	5	0	48	10	63	73
Δ*adhE*	0	11	62	0	0	21	73	94
Engineered strains for the production of ethanol and co-products. All have the plasmid pZSKLMgldA								
SY03	79	0	2	12	6	11	87	98
SY04	80	0	2	75	4	9	85	94
EH05 60 g/L gly.	84	1	4	9	0	5	89	94

[a] Unless stated, cells were cultivated in medium and conditions described in *Materials and Methods*. Data represent the average of three samples taken at the earlier of the time at which: (i) a culture of the specific strain entered stationary phase or (ii) the BW25113 culture entered stationary phase. The coefficient of variation was below 9% in all cases. Two asterisks (**) indicate that the inoculum was prepared in minimal medium supplemented with 5 g/L yeast extract and 10 g/L tryptone and the initial fermenter OD550 was ~ 0.5.

[b] Carbon recovery is expressed as % mol of carbon in product, including biomass, per mol of carbon in glycerol consumed. The column "product" shows the total recovery of carbon in products, assuming that mol of acetate *plus* mol of ethanol *equals* mol of 1-C compounds (formate *plus* CO_2) generated by the dissimilation of pyruvate. The column "overall" shows the overall carbon recovery, including products and biomass.

The extremely high ratio of oxygen to cell mass in the initial phase of culture led us to hypothesize that the activity of the GlpK-GlpD pathway might be required for the almost completely aerobic conditions found in the beginning of the fermentation. To test the hypothesis, the standard MOPS minimal media inocula was augmented with 5 g/L yeast extract and 10 g/L tryptone. Once the inocula had grown sufficiently, they were washed with MOPS minimal medium in order to avoid carry over of rich nutrients and then added to the fermenters in both high and low initial OD_{550} of 0.05 and 0.5, respectively. In a situation similar to the GldA-DHAK pathway, the Δ*glpK* and Δ*glpD* mutants had similar growth curves and glycerol consumption, although the high and low inocula conditions were different (Fig. 8, data not shown).

The high inocula cultures grew with no detectable lag phase, although cell growth and glycerol consumption for both mutants were significantly impaired: 60% and less than 75%, respectively, of that observed for wild-type BW25113 (Fig. 8). The volumetric rate of glycerol consumption was even lower, as both mutants had rates less than 60% of the wild-type (data not shown). While mutants lacking the GldA-DHAK pathway were able to utilize the oxygen in the microaerobic environment for reducing equivalents, the Δ*glpK* and Δ*glpD* mutants could not because the enzymes are mostly

inactive with high amounts of oxygen. GldA-DHAK pathway mutants had a much higher acetate/ethanol ratio 1.54, relative to that of BW25113 (0.24). Meanwhile, Δ*glpK* and Δ*glpD* mutants had an acetate/ethanol ratio of less than 0.16 (Durnin et. al. 2009). While the larger inocula cultures had no lag phase, the cultures with small inocula had a significant one. In this case, the mutants grew very slowly for about 24 hours, at which point they reached an OD_{550} of about 0.12 (data not shown). As cells grew in a more anaerobic environment for the next 24 hours, OD_{550} increased to 0.8; in this time span, the low inoculum flask showed as much cell growth as the high inoculum flask (data not shown). As can be expected, cell growth and glycerol fermentation were significantly impaired in these cultures, with glycerol consumption less than 20% of the BW25113 wild type (Fig. 8). Taken together, these results indicate that the GlpK-GlpD contributed to the efficiency of microaerobic growth on glycerol, but was not essential for this metabolic process especially if the oxygen/cell mass ratio is not too high (Durnin et. al. 2009). Even with initial starting oxygen to cell mass ratio of 130 mg/L/OD, these mutants could grow. Moreover, when the GldA-DhaKLM pathway was overexpressed in strain Δ*glpK* or Δ*glpD*, cell growth and glycerol fermentation occurred at levels equivalent or better than those observed for wild-type BW25113 (Fig. 8). This mutant was able to thrive in

both the initial high oxygen phase and the mostly anaerobic phase. It produced more ethanol than BW25113 with an acetate/ethanol ratio of less than 0.09 and consumed 50% more glycerol (Durnin et. al. 2009).

In conclusion, both the GldA-DhaKLM and GlpK-GlpD pathways play a significant role in the conversion of glycerol to the glycolytic intermediate DHAP, while the GldA-DhaKLM pathway becomes dominant once the oxygen/cell mass ratio is low. Most of the DHAP is catalyzed first to phosphoenolpyruvate (PEP) and then to pyruvate via common glycolytic enzymes to complete the Embden-Meyerhof-Parnas (EMP) pathway while some enters gluconeogenic metabolism to produce several 4-, 5-, and 6-C precursor metabolites such as fructose-6-phosphate, glucose-6-phosphate and pentose phosphate pathway intermediates (Romeo & Snoep 2005). The enzymes required for gluconeogenic metabolism, such as fructose bisphosphate aldolase, fructose 1,6-bisphosphatase, and phosphoglucose isomerase, are required for the growth of *E. coli* on C-3 carbon sources such as glycerol (Romeo & Snoep 2005). Our research found a similar requirement in microaerobic environments (data not shown).

4.4. Role of pathways involved in the synthesis of metabolic products originating from phosphoenolpyruvate, pyruvate, and acetyl-CoA

The metabolism of phosphoenolpyruvate (PEP) and pyruvate resulted in varying degrees of energy production and reduction potential depending on the mixture of different metabolic products generated such as succinate, lactate, acetate, ethanol, and formate (Fig. 3). Succinate is the only product derived straight from PEP without going through the pyruvate pathway, and in doing so it produces a small amount of energy through the proton motive force and two reducing equivalents ((Lawford & Haddock 1973, Gennis & Stewart 1996). We evaluated the mutant Δ*frdA*, which lacks the enzyme fumarate reductase (FRD). FRD is involved in final step of the primary route responsible for succinate synthesis (Sawers & Clark 2004). Previous research (data not shown) with the mutant in anaerobic conditions led us to believe strain Δ*frdA* would survive in a microaerobic environment, and our research confirmed this as it grew and utilized glycerol at levels similar to those observed in strain BW25113 (Fig. 9) while producing negligible amounts of succinate (Table 4). Interestingly, not only did this mutant have a similar glycerol utilization rate to BW25113, it also had a similar acetate to ethanol ratio (Durnin et. al. 2009). While the succinate pathway does not include pyruvate, it is a precursor to all the other product pathways. Four

different enzymes metabolize pyruvate: lactate dehydrogenase (LDH, *ldhA*), pyruvate formate lyase (PFL, *pflB*), pyruvate dehydrogenase (PDH), and pyruvate oxidase (POX, *poxB*). While LDH catalyzes the reduction of pyruvate to lactate, we did not study its role in the microaerobic utilization of glycerol because we found no lactate in the wild-type BW25113 fermentation (Fig. 9, Table 4).

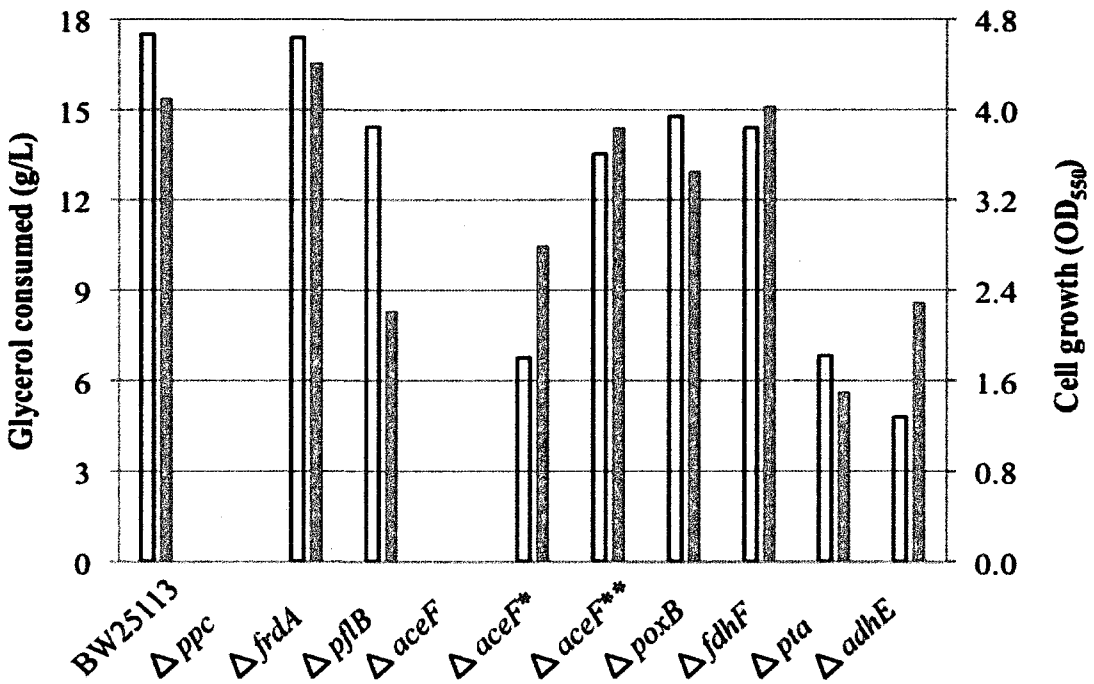


Figure 9. Effect of inactivation of pathways involved in the synthesis of metabolic products originating from phosphoenolpyruvate, pyruvate, and acetyl-CoA. Glycerol utilization (clear bars) and cell growth (gray bars) are shown. Unless otherwise specified, values represent the means for three samples taken at the earlier of: (i) the time at which a culture of the specific strain entered stationary phase or (ii) the time at which the BW25113 culture entered stationary phase. Coefficient of variation was below 7.52% in all cases. One asterisk (*) means the inoculum was grown in LB. Two asterisks (**) mean a high (10x) inoculum as well as being grown in LB. Figure originally in Durnin et. al. 2009.

There are two enzymes that mediate the production of acetyl-CoA from pyruvate: PFL, encoded by *pflB*, and PDH, encoded by the *aceEF-lpdA* operon. These two enzymes operate at different oxygen concentrations. PFL, which is extremely oxygen sensitive, is primarily active during anaerobic fermentation, although it has been reported to be active during microaerobic metabolism of sugars (Sawers & Clark 2004). PDH is predominantly active in the presence of oxygen or other electron acceptors, although low levels of PDH activity have been reported under fermentative conditions (Sawers & Clark 2004, Gonzalez et. al. 2008). While both pathways lead to the production of acetyl-CoA, PDH produces reducing equivalents while the PFL pathway does not. Instead, pyruvate is metabolized to formate. This indicates that the redox status of the cell determines which of the two enzymes, PDH or PFL, is active under a particular condition. If the PDH pathway was active in anaerobic conditions, reduction potential would build up, and glycerol consumption would stop. The PFL pathway was inactive in aerobic environments because external electron acceptors consumed the reducing equivalents. As dissolved oxygen was detectable in the fermentation broth only for the initial 2 hours of the cultures (Fig. 5B), it was expected that PDH would be more active during the initial stages of the fermentation while PFL would be the

primary enzyme for the rest of the fermentation. Several lines of evidence support this hypothesis. First, as in the case of both the strains Δ*glpK* and Δ*glpD*, the inoculum of a PDH mutant Δ*aceF* required complex supplementation (5 g/L yeast extract and 10 g/L tryptone) for any detectable growth. Both high and low initial biomass fermentations with Δ*aceF* were run to see how this might affect glycerol consumption. The low initial OD_{550} strain exhibited very slow growth during the early stages- less than two doublings of the cell mass in 15 hours. Soon after this, the low inoculum fermentation of Δ*aceF* had very similar growth characteristics to the BW21533 wild type (data not shown). Nevertheless, due to the initial 15 hours, there was a significant reduction in glycerol utilization and cell growth overall: only 53% and 68%, respectively, of that observed for BW25113 once the fermentation was completed (Fig. 9). Significantly more acetate and less ethanol were produced by this strain as it struggled to grow in an oxygen rich environment, with an acetate/ethanol ratio of 0.48 at the end of the fermentation (data not shown). Most of the ethanol was only synthesized near the end of the fermentation; acetate was the primary product while the oxygen to cell mass ratio was high (data not shown). When the fermenter was inoculated with a higher starting OD_{550} of Δ*aceF*, the growth curve was much smoother, leading to higher cell mass production

and glycerol consumption compared to the low inoculum fermentation (92% and 77%, respectfully, recorded in wild-type BW25113 in Figure 9). It is noteworthy that the composition of the fermentation products for the high initial cell mass experiment of Δ*aceF* was relatively similar to that of BW25113 (Table 4). As the high inoculum fermentation required less acetate for growth, the acetate/ethanol ratio was only 0.16 (Durnin et. al. 2009). Overall, the Δ*aceF* strains grew very poorly with high oxygen/cell ratios and started to act more like wild type once the ratio had significantly decreased. It appears that a sufficiently large initial biomass could lower the oxygen to cell ratio for near wild type growth. The PFL-deficient strain (Δ*pflB*) exhibited the exact opposite behaviors. For the first 32 hours of fermentation, both cell growth and glycerol consumption were the same as wild-type, after which Δ*pflB* entered into an "early" stationary phase and stopped cell growth (data not shown). This resulted in a reduction of cell growth by 54% and glycerol fermentation by 82% compared to the wild-type BW25113 fermentation (Fig. 9). More importantly, a significant redistribution of fermentation products took place in response to the *pflB* mutation: strain Δ*pflB* exhibited an almost homolactic behavior, producing about 11.5 g/L of lactate while producing very little ethanol or acetate, whereas wild type generates undetectable amounts of lactate (Table 4).

Taken together, these results show that although both PFL and PDH are involved in the conversion of pyruvate to acetyl-CoA under microaerobic conditions, PFL is responsible for a larger fraction of the flux through this step due to the inability of the PDH pathway to operate in a mostly anaerobic environment. Furthermore, these enzymes appear to have complementary roles during early (PDH) and late (PFL) stages of cell growth, perhaps due to the transition from an "aerobic-like" to an "anaerobic-like" environment as the fermentation proceeds.

The enzyme POX, encoded by *poxB*, which generates acetate and CO_2 from pyruvate through an alternative acetate pathway, has a significant part in pyruvate metabolism and is important for the growth efficiency of wild-type *E. coli* under aerobic conditions (Li et. al. 2007; Abdel-Hamid et. al. 2001). However, when we tested a *ΔpoxB* strain in microaerobic conditions it exhibited only a small decrease in cell growth and glycerol fermentation (~ 15% for both, Fig. 9), and no significant redistribution of carbon among fermentation products (Table 4). When one of the primary acetate pathways such as PDH or PFL was cut off, a significant portion of carbon that would otherwise be metabolized into acetate instead formed other products because the POX pathway was unable to make up for either the PDH or PFL pathways in either partially aerobic or anaerobic conditions (Fig. 9).

Because the PDH pathway produces reducing equivalents and CO_2 when metabolizing pyruvate, it is optimal in environments with exterior electron acceptors. PFL, on the other hand, conserves the reducing equivalents with the generation of formate. The formate becomes the ultimate end product and can be secreted to the extracellular medium (generally done in basic environments to help regulate the pH) or else is converted to CO_2 and hydrogen by the action of the enzyme formate hydrogen-lyase (FHL) (Sawers et. al. 2004). Our lab has recently reported that FHL is required for the fermentative metabolism of glycerol at acidic pH, so we decided to continue the investigation to see if FLH is active in the microaerobic utilization of glycerol (Gonzalez et. al. 2008).

The mutant Δ*fdhF* (*fdh* codes for FHL) grown in a microaerobic environment had similar growth curve and glycerol consumption as the wild-type strain (Fig. 9) but had a very different composition of metabolic products (Table 4). Δ*fdhF* produced about 0.4 g/L of lactate and had a significantly higher acetate/ethanol ratio of 0.67 as acetate production replaced that of ethanol, compared to wild-type BW25113 with undetectable lactate levels and a ratio of 0.28 (Table 4). We hypothesize that FHL inactivation leads to the synthesis of products with net increase in reducing equivalents from glycerol as the cell tries to compensate for a low

$NADH/NAD^+$ ratio. This internal redox state is caused by the lack of hydrogen generation and recycling that FHL regulates. The literature has thoroughly documented the metabolic recycling of the hydrogen produced by FHL, the consequent increase in $NADH/NAD^+$ ratios and the enzyme's impact on the synthesis of reduced and oxidized products (Clark 1989, Sawers & Watson 1998, Sawers & Clark 2004, Sawers et. al. 2004, Murarka et. al. 2008).

Acetate can either be generated from pyruvate via the POX pathway or from acetyl-CoA through the phosphate acetyltransferase (PTA)-acetate kinase (ACK) pathway (Sawers & Clark 2004). As mentioned above and shown in Figure 9, the POX pathway appeared to play a minimal role in the metabolism of glycerol under microaerobic conditions. However, inactivation of the PTA-ACK pathway had a significant impact on glycerol utilization and cell growth as demonstrated by the strain Δ*pta* (Fig. 9, *pta* codes for PTA). Acetate synthesis was reduced to ~1/10 of the levels produced by the wild-type, demonstrating the ACK-PTA pathway's importance (Table 4). In addition to the almost complete removal of acetate, strain Δ*pta* also entirely changed the product selection; it exhibited an almost homolactic behavior, producing almost 3 g/L of lactate (~50% w/w yield respect to the glycerol consumed) compared to undetectable amounts in the

wild type (Table 4). This amount of lactate was more than 10 times the amount of any other product synthesized by strain Δ*pta* (w/w basis). These results for microaerobic glycerol consumption contrast with those reported for the fermentative utilization of glycerol, in which case the inactivation of the PTA-ACK pathway had almost no effect on glycerol fermentation (Murarka et. al. 2008).

Ethanol can be converted from acetyl-CoA in a two-reaction pathway that utilizes acetaldehyde as the intermediate. The entire process from acetyl-CoA to ethanol occurs due to one enzyme- alcohol/acetaldehyde dehydrogenase (ADH) coded by *adhE* (Sawers & Clark 2004). The ADH pathway is the primary pathway for the microaerobic utilization of glycerol by BW25113 wild type (Figs. 4-6). While there are external electron acceptors in this environment, we hypothesized that the ratio of oxygen/cell mass was low enough that ethanol must be utilized for redox balance due to the highly reduced state of carbon in glycerol. To assess the relevance of this hypothesis, we utilized the mutant *ΔadhE*, which we confirmed was devoid of ADH activity (activity in *ΔadhE* was undetectable compared to 0.0306 mmol/gcell/min in BW25113) (Durnin et. al. 2009). Without any ADH activity, the strain produced no ethanol (Table 4) and had significantly impaired cell growth and glycerol utilization with both less than 40% of the

wild type (Fig. 9). Even with a relatively large percentage of external electron acceptors, ethanol synthesis plays a dominant role in the microaerobic utilization of glycerol.

4.5. Carbon flux for the microaerobic fermentation of glycerol

Figure 10 illustrates the pathways involved in the microaerobic utilization of glycerol by *E. coli*, as concluded from the genetic and biochemical evidence collected for this report and explained above. The arrow thickness is a graphic depiction of the magnitude of the carbon flux through the various pathways. The enzyme assays and mutant strain fermentations demonstrate both the respiratory (GlpK-GlpD) and fermentative (GldA-DHAK) pathways play a significant role in the conversion of glycerol to glycolytic intermediate dihydroxyacetone (Fig. 8, Table 3). Both pathways are important for growth under microaerobic conditions. While each pathway is predominant during different stages of the fermentation depending on the NADH/NAD^+ ratio, either pathway can support growth and glycerol utilization at a similar level to the wild type if it is expressed at sufficient levels. Pyruvate dehydrogenase (PDH) and pyruvate formate-lyase (PFL) are another set of competing pathways that appear to compliment each other: PDH operates in respiratory conditions

and PFL is active in anaerobic ones. Evidence from both this study and others in our lab supports the conclusion that PFL is the primary pathway in both anaerobic and microaerobic conditions (Murarka et. al. 2008). The mutant *ΔpflB* had very little carbon flux to acetate and ethanol (Table 4). It instead generated lactate from glycerol, which signifies a bottleneck at the pyruvate node (Durnin et. al. 2009). While strain *ΔaceF* did show a significant decrease in glycerol consumption and cell growth (Fig. 9), the fermentation product mix stayed very similar to that of the wild-type, showing similar carbon flux through the pyruvate node (Table 4). Lastly, the engineered strain SY04 (pZSKLMgldA) primarily synthesized ethanol and formate in almost equimolar amounts (Table 4, Fig. 11C). This data proves that almost all the pyruvate was metabolized via PFL (Durnin et. al. 2009). PFL activity is regulated by both the acetate and ethanol pathways. When either one of these is blocked, there are significant changes in product composition. However, the other various pathways that produce metabolic products, such as FrdA, LdhA and PoxB, do not appear to play a significant role in the microaerobic utilization of glycerol (Fig. 8, Fig. 9, Table 4).

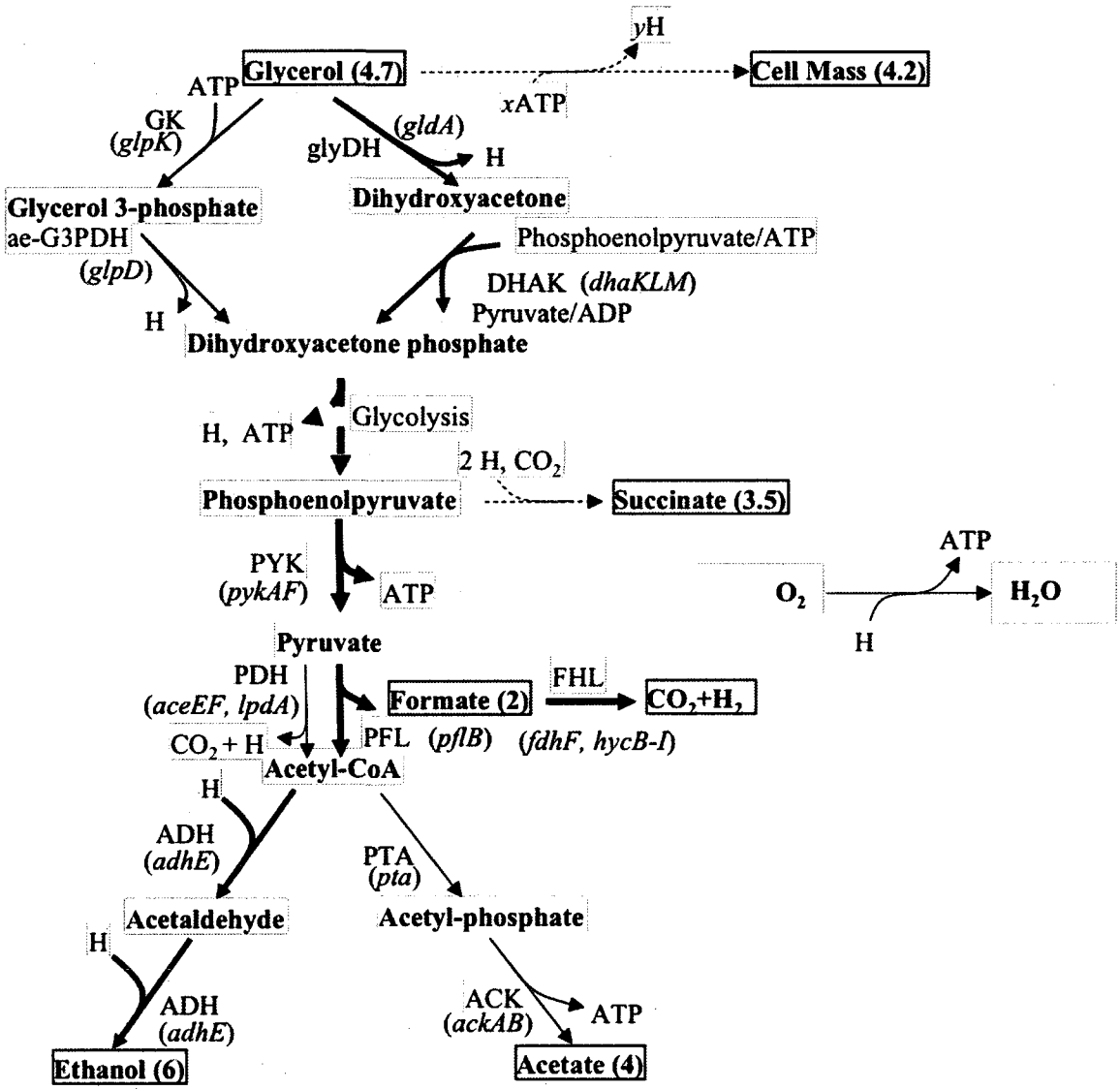


Figure 10. Pathways involved in the generation of glycolytic intermediates and the synthesis of metabolic products during the microaerobic utilization of glycerol. Abbreviations: Broken lines illustrate multiple steps. The thickness of the lines represent the relative contribution to the carbon flux. Extracellular metabolites are boxed. Figure originally in Durnin et. al. 2009.

4.6. Engineered strains for the conversion of glycerol to ethanol under microaerobic conditions.

Our research group previously published on strains of *E. coli* that are engineered for the anaerobic metabolism of ethanol from glycerol (Yazdani & Gonzalez 2008). These strains have a very high ethanol recovery since they utilize the reduction potential of each highly reduced glycerol carbon. Two secondary products, formate and hydrogen, can be produced depending on additional genetic engineering. The two strains in the paper SY03 (pZSKLMgldA) and SY04 (pZSKLMgldA), produced ethanol & hydrogen and ethanol & formate, respectively. We optimized ethanol production in these strains by inactivating the enzymes that synthesize succinate and acetate through gene deletions, Δ*frdA* (fumarate reductase) and Δ*pta* (phosphate acetyltransferase), respectively. The strain SY04, which produced both ethanol & formate, had another mutation to delete the gene *fdhF* that blocked formate-hydrogen lyase, and thus prevented the conversion of formate to CO_2 and H_2 (Durnin et. al. 2009). The carbon flux pathways are shown in the Appendix A. High rates of glycerol utilization and product synthesis were achieved with the simultaneous overexpression of glycerol dehydrogenase (*gldA*) and dihydroxyacetone kinase (*dhaKLM*), which are the enzymes responsible for the conversion of glycerol to

glycolytic intermediate dihydroxyacetone phosphate under fermentative conditions (Durnin et. al. 2009). Although *E. coli* has a redox balanced pathway from glycerol to ethanol, it is unable to survive in a fermentative environment without rich nutrients such as tryptone, even with these genetic mutations (Yazdani & Gonzalez 2008). As the wild-type can generate reduced products under microaerobic conditions using a minimal medium without tryptone supplementation, we decided to evaluate the performance of strains SY03 (pZSKLMgldA) and SY04 (pZSKLMgldA) (Fig. 11, panels B and C). Both strains produced ethanol yields that were just below 80% of the total carbon consumed and more than 90% of product production. SY03 (pZSKLMgldA) and SY04 (pZSKLMgldA) both produced ethanol very quickly, at rates of 4.45 and 4.89 mMol/L/h, which are better performances than that reported in an anaerobic environment where the strains produced 0.94 and 3.58 mMol/L/h, respectively (Durnin et. al. 2009, Yazdani & Gonzalez 2008). The strains in anaerobic conditions used less carbon for cell mass due to fewer outside electron acceptors and a 2 g/L tryptone supplement. Nevertheless, the specific ethanol rates for SY03 (pZSKLMgldA) and SY04 (pZSKLMgldA) in microaerobic conditions of 9.55 and 10.52 mMol/g cell/h still compared favorably to the anaerobic data of 16.02 and 8.16 mMol/g cell/h, respectively (Durnin et. al. 2009, Yazdani

& Gonzalez 2008). In both cases, the engineered strains did much better than the wild type MG1655 (Fig. 10A); in microaerobic conditions only 52% of the total carbon is metabolized into ethanol at a volumetric rate of 2.02 mMol/L/h and specific rate of 4.73 mMol/g cell/h.

While both the SY03 and SY04 strains have twice the ethanol synthesis rate of the wild-type, they produced relatively significant amounts of lactate (6 and 4%, respectively) which would be problematic at higher glycerol levels (Table 4). Inactivation of fermentative lactate dehydrogenase (LdhA, coded on *ldhA*), the enzyme that metabolizes pyruvate to lactate, would make more carbon available for ethanol synthesis while removing lactate from the fermentative broth, making ethanol concentration cheaper. We deleted the gene *ldhA* in both the SY03 and SY04 strains leading to two new mutants, EH05 (SY03Δ*ldhA*) and EF06 (SY04Δ*ldhA*) that also had the plasmid pZSKLMgldA (Durnin et. al. 2009). The carbon flux is shown in Appendix A. These two strains produced no lactate and had higher ethanol yields- both more than 95% of product produced (Durnin et. al. 2009). While EH05 (pZSKLMgldA) continued to have similar ethanol production rates of 4.11 mMol/L/h, the SY04 derivative produced much less at only 2.62 mMol/L/h.

The specific rates are similar with EH05 (pZSKLMgldA) producing about the same ethanol per gram of cell per hour as that of SY03 (pZSKLMgldA), and EF06 (pZSKLMgldA) underperformed comparably (9.87 and 6.72 mMol/g cell/h, respectively). When EH05 (pZSKLMgldA) was cultured on high glycerol concentrations (60 g/L), it continued to perform well and had a total conversion rate of 83% of carbon to ethanol and 95% of all products produced (Table 4). The strain continued to produce ethanol at a high volumetric rate of 4.89 mMol/L/h, although the specific rate never surpassed 7.02 mMol/g cell/h. Figure 10D shows a representative example of ethanol production compared to other products and demonstrates that the strain EH05 can produce ethanol at high rates with higher glycerol concentrations. The data reported here shows this genetically engineered strain is superior to others reported in the literature for the conversion of glycerol to ethanol by other microorganisms and similar to *E. coli* strains engineered to use cellulosic sugars (Jarvis et. al. 1997, Ito et. al. 2005, Sakai & Yagishita 2007, Underwood et. al. 2002). Strain EH05 (pZSKLMgldA) produced hydrogen as well as ethanol, which implied an overall higher yield and rate of product synthesis of that reported for the production of ethanol from sugars (Durnin 2009). Small amounts of 1,2-PDO showed up in the high glycerol fermentation, most likely due to the near anaerobic conditions

faced by the strain (data not shown). Strain EF06 (pZSKLMgldA) continued to lag the SY03 derivative, most likely due to the large accumulation of formate and ethanol (data not shown, Yoshida et. al. 2006).

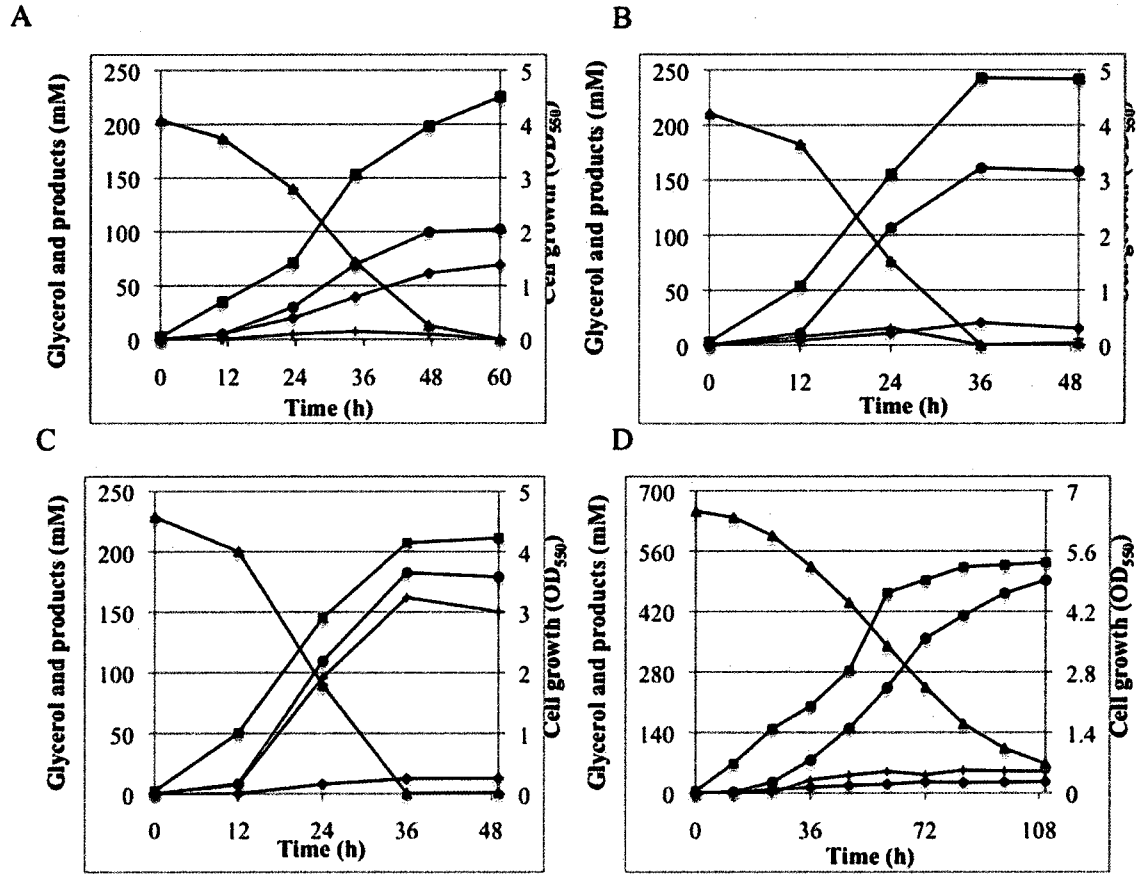


Figure 11. Synthesis of ethanol and co-products by strains MG1655 **(A)**, SY03 (pZSKLMgldA) **(B)**, SY04 (pZSKLMgldA) **(C)**, and EH05 (pZSKLMgldA) **(D)**. Data are shown for concentration of cells (■), glycerol (▲), ethanol (●), formate (*), and other products (◆). The coefficient of variation was below 8% in all cases. Figure originally in Durnin et. al. 2009.

5. Conclusion

Biofuels have become more attractive in the past few years as governments worry about pollution and fuel security. In the process, biodiesel capacity has increased by billions of gallons throughout the world, creating vast quantities of glycerol that have not been used to their potential. The price, availability and reduced state of the carbon of glycerol have made it a very attractive choice for use as substrate in fermentation to produce highly reduced fuels and chemicals such as ethanol (Yazdani & Gonzalez 2007). However, until recently the fermentative metabolism of glycerol had been reported in only a few species of the genera *Klebsiella*, *Citrobacter*, *Enterobacter*, *Clostridium*, *Lactobacillus*, *Bacillus*, *Propionibacterium*, and *Anaerobiospirillum* that did not lend themselves well to engineering (Yazdani & Gonzalez 2007 and references cited therein).

Nevertheless, our research group recently discovered *E. coli* is able to metabolize glycerol in a fermentative manner utilizing 1, 2 PDO as shown in Figure 4B (Dharmadi et. al. 2006, Gonzalez et. al. 2008, Murarka et. al. 2008). *E. coli* is much easier to engineer than other glycerol subsisting species and our research group has succeeded in optimizing ethanol yield with both pure and crude glycerol (Yazdani & Gonzalez 2008). However, the process is relatively expensive because it requires the use of rich

nutrients to enable redox balance for cell mass production (Gonzalez et. al. 2008, Murarka et. al. 2008). This study explored the use of limited amounts of oxygen to be used as an electron acceptor, which would enable redox balance by consuming the excess reducing equivalents generated by the incorporation of glycerol into cell mass. We found that microaerobic conditions eliminated the need for rich nutrients while producing more cell mass and a high percentage of reduced products. We confirmed that several respiratory pathways (with genes *aceF*, *glpK*, *glpD*) were required for initial growth. However, once a sizeable cell mass was available, these mutants were able to grow, albeit slower than the wild type. Our hypothesis about genes that could be deleted with few repercussions (*fdhF, frdA, poxB*) was correct, this demonstrated that the FDH, FRD and PoxB pathways were not required grow. These results allowed for better understanding of how *E. coli* grow microaerobically and how best to genetically engineer strains. The strains discussed in Yazdani & Gonzalez (2008) were tested and improved to achieve high yields and productivities of ethanol with the co-products hydrogen and formate (Fig. 11).

We are currently extending our approach to engineer *E. coli* for the production of other chemicals and fuels in a minimal medium with no rich supplements. Figure 12 presents examples of other products that could be

synthesized from glycerol in microaerobic conditions as they are produced via either redox-balanced or redox-generating pathways. The small amounts of oxygen available in microaerobic conditions allow for redox balance in the glycerol to cell mass pathway, which means the theoretical maximum yield in each case is higher than that possible with the use of common sugars such as glucose or xylose. This higher yield means that glycerol should achieve a similar price as that of glucose. This should increase biodiesel profit margins, allowing for further expansion in biodiesel capacity.

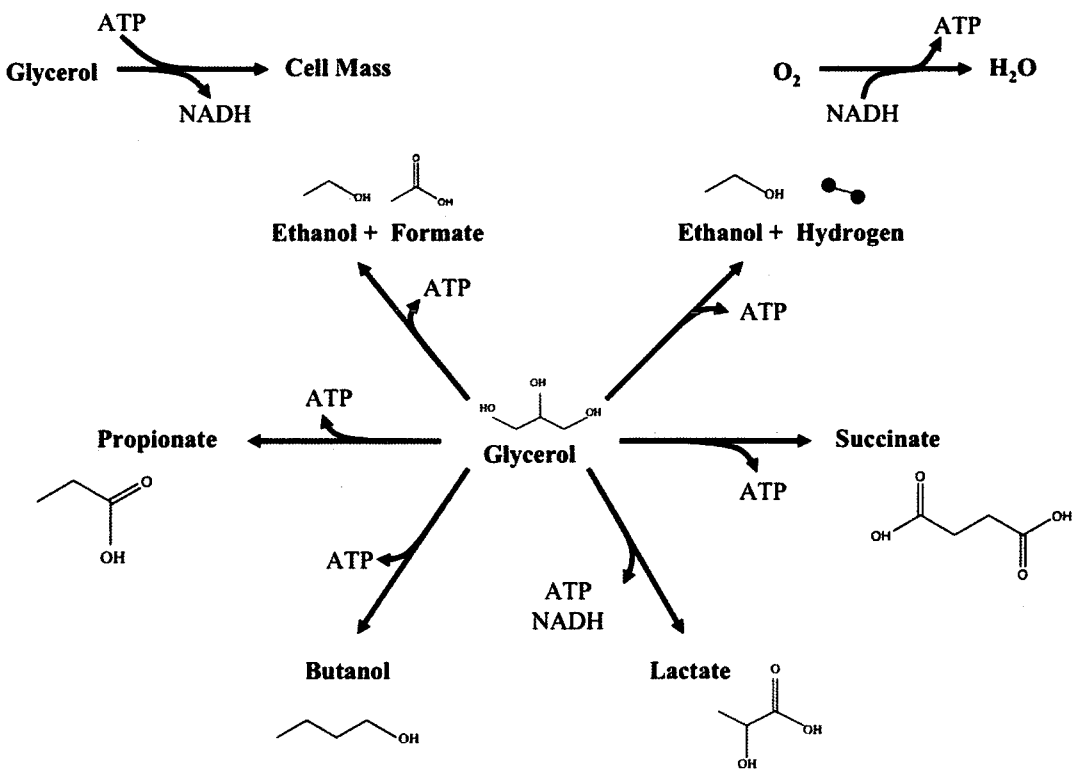
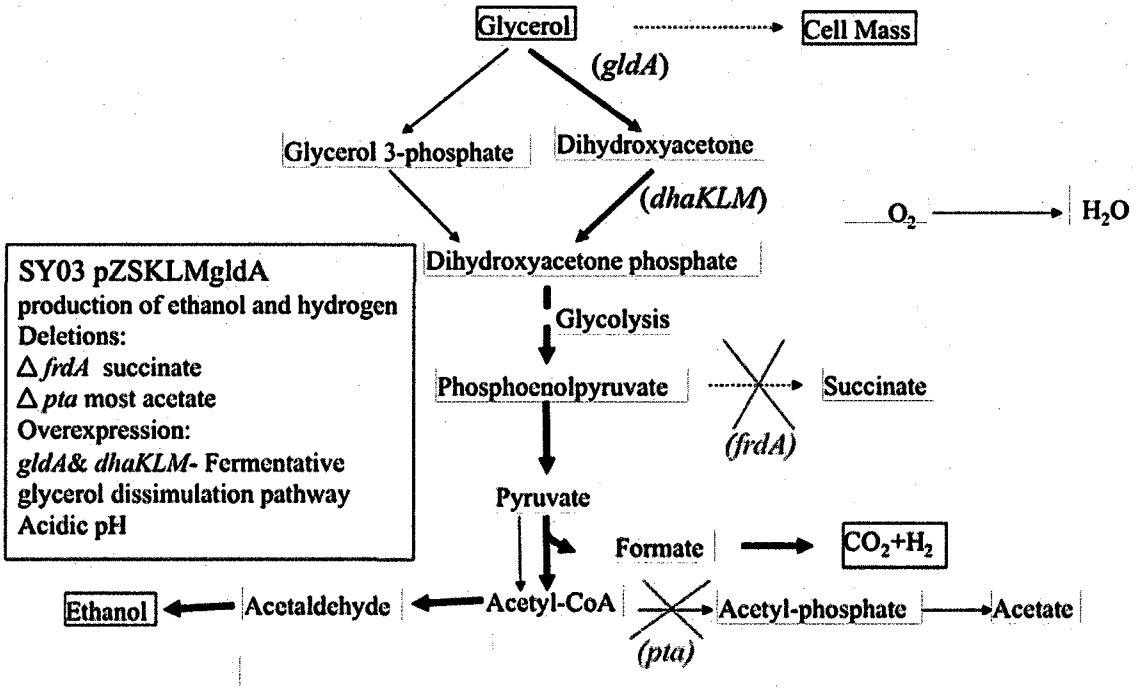
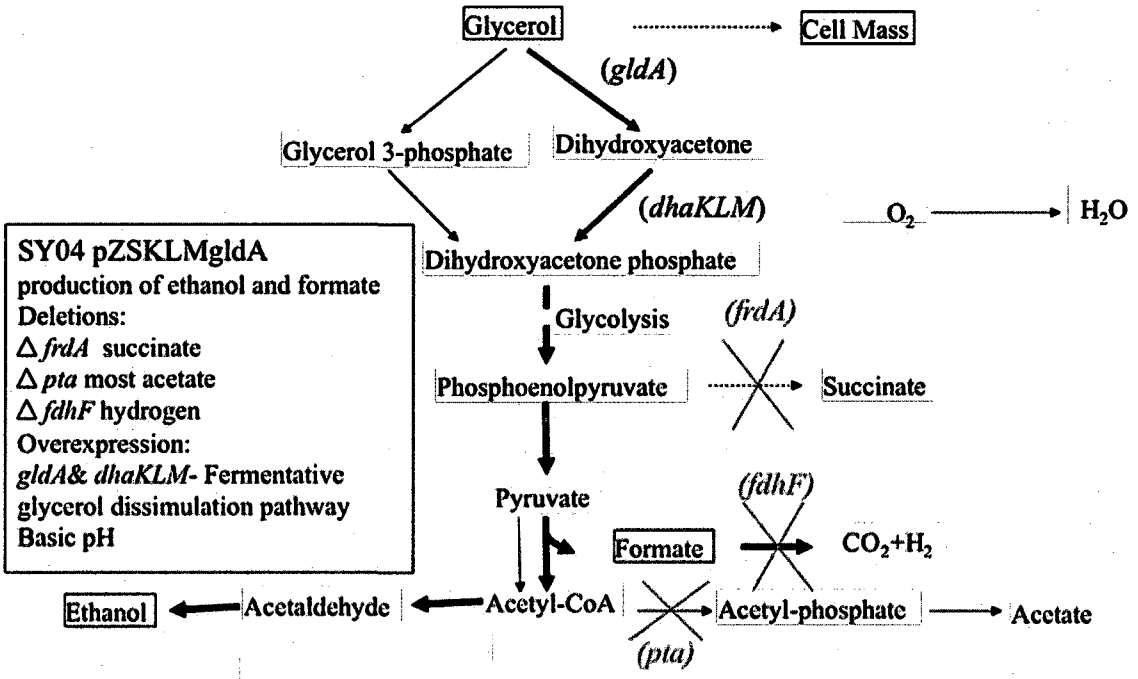


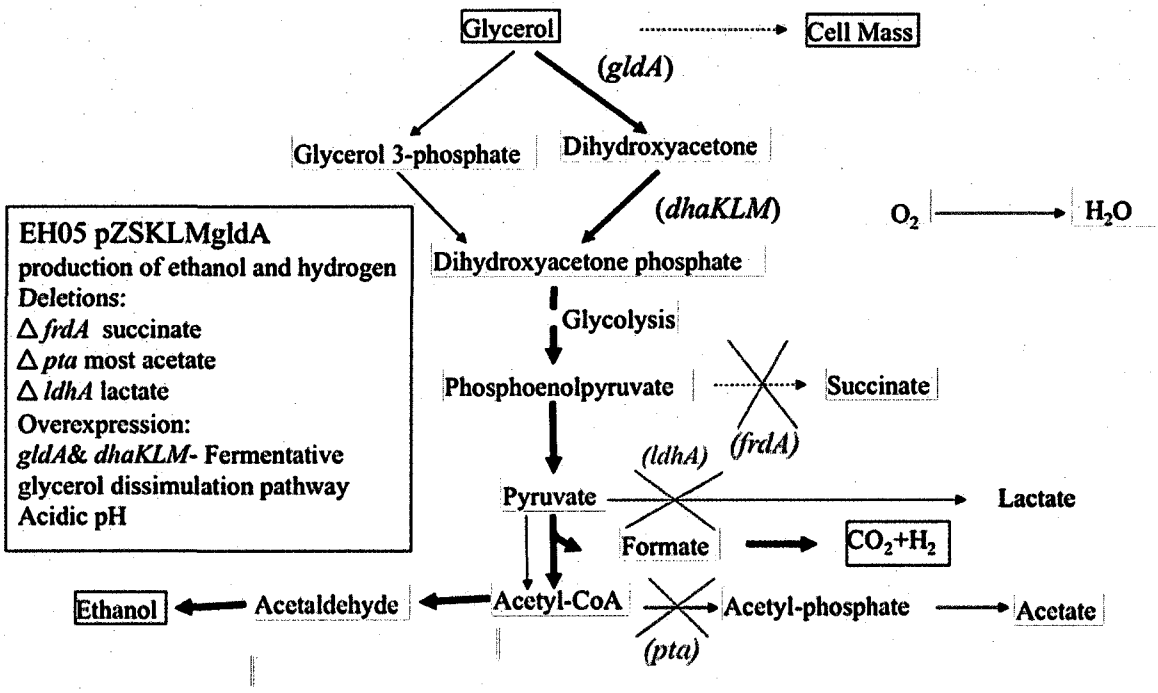
Fig. 12. Microaerobic metabolism of glycerol as a platform for the production of fuels and reduced chemicals. Abbreviations: AcCoA, acetyl-coenzyme-A; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate.

6. Appendix A

Carbon Flux diagrams showing the pathways and gene manipulations for the various engineered strains SY03, SY04 and EH05.






Glycerol
Cell Mass
(gldA)
Glycerol 3-phosphate
Dihydroxyacetone
(dhaKLM)
O2
H2O
EH05 pZSKLMgldA
production of ethanol and hydrogen
Deletions:
Δ frdA succinate
Δ pta most acetate
Δ ldhA lactate
Overexpression:
gldA& dhaKLM- Fermentative
glycerol dissimulation pathway
Acidic pH
Dihydroxyacetone phosphate
Glycolysis
Phosphoenolpyruvate
Succinate
(frdA)
(ldhA)
Pyruvate
Lactate
Formate
CO2+H2
Ethanol
Acetaldehyde
Acetyl-CoA
Acetyl-phosphate
Acetate
(pta)

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